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The Effects of ION*Gut Health Dietary Supplement on Markers of Intestinal Permeability and Immune System Function in Healthy Subjects; A Double-Blind, Placebo-Controlled Clinical Trial

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Overview

Intestinal permeability is an emerging paradigm in the scientific and clinical understanding of human health and disease. The understanding of acute and chronic inflammation in animal and human health and disease has been well established for many decades. However, the role of intestinal and blood-brain barrier permeability as an important predictor of these inflammatory events, which impact longevity and health, has only entered mainstream medical science in the last decade. The functional anatomy of the intestinal lining and adjacent immune system is comprised of four elements: the intestinal mucosal barrier (epithelial layer); the adjacent immune system composed of the gut-associated lymphoid tissue (GALT) and the glial-cell mediated immune system of the peripheral nervous system; the enteric endocrine system (neurotransmitter production); and the autonomic nervous system components such as the enteric afferent sensory network, afferent and efferent pathways of the parasympathetic (relaxation, digestion, metabolism) and sympathetic (fight-or-flight) nervous system.

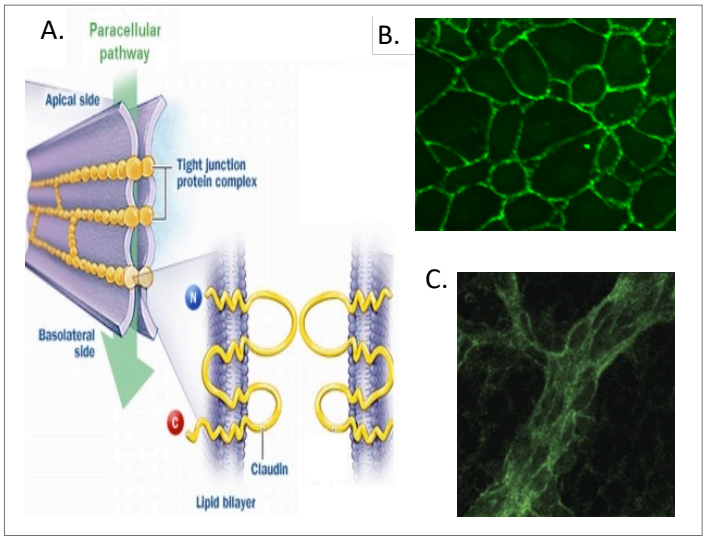

Figure 1. Tri-layer Network of Tight Junctions composed of transmembrane and extracellular protein complexes (Panel A). Immunohistochemistry Images of small intestine epithelial (IEC6) barrier (Panel B). Vascular endothelial barrier of the arterial, venous, and capillary vessels (Panel C).

Intestinal permeability describes the function or dysfunction of a network of protein interactions, including the tight junction proteins, which regulate the passage of nutrients, pathogens, and other macro-molecules across the intestinal and blood-brain barriers, which are comprised of billions of adherent epithelial and endothelial cell populations, respectively. The tight junction is an aggregate protein-complex comprised of multiple protein structures located within the phospholipid bilayer of adjacent intestinal epithelial or vascular endothelial cell walls (Figure 1). This multi-layer protein network is typically located near the apical aspect of the epithelial or endothelial barriers, and functions as 'intelligent' gatekeepers to the entry or block of nutrients, toxins, and microbes.

Tight junction integrity has been shown to be vulnerable to damage from a variety of natural compounds such as gluten and alcohol, and synthetic herbicides such as glyphosate (Roundup®), and may also be affected by some pharmaceutical compounds, including some vaccines, non-steroidal anti-inflammatories, and the laxative MiraLAX®. Disruption of the intestinal epithelial tight junction system can then lead to dysregulation of the intestinal and blood-brain barrier, causing the

immune system of the gut (GALT) and the glial cells of the nervous system to be overwhelmed by the influx of foreign material.

Our previously-published, peer-reviewed basic science studies have demonstrated the in vitro beneficial effects of the novel aqueous humic extract supplement – ION*Gut Health® - on tight junction integrity in healthy and damaged epithelium cell populations exposed to gluten, and/or glyphosate. Our in vitro studies demonstrated that ION*Gut Health® has unique biologic effects on human cell genomic transcription of functional tight junction proteins, mitochondrial oxidative stress, and anti-oxidant production.

The current double-blind, placebo-controlled clinical study was performed to evaluate the effects of this aqueous humic extract as a daily, oral dietary supplement on urine markers of intestinal permeability and immune system function in healthy subjects with varied American-diet lifestyles. The US food system has been recognized to have a ubiquitous component of glyphosate (Roundup®) residues that can have adverse effects on the intestinal microbiome diversity and volume, as well as tight junction function and intestinal permeability long before systemic disorder or disease develops.

Urine Markers

Inflammatory cytokines. Tumor necrosis factor alpha (**TNFα**, cachexin, or cachectin) and interleukin-6 (IL-6) are major pro-inflammatory cytokines involved in cell signaling of inflammation and considered an acute phase reactant. TNFα is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. The primary role of TNFα is the regulation of immune cell response, including fever induction, apoptotic cell death, and cachexia (muscle loss) which work to inhibit tumorigenesis, viral replication and bacterial sepsis. The measurement of urine TNFα in the healthy subjects of the present study functioned as a screening for occult acute inflammatory processes.

Interleukin 6 **(IL-6)** is an immune-system modulating protein produced and secreted by macrophage, glial cells, and smooth muscle cells in response to non-human molecules referred to as pathogen-associated molecular patterns which bind to a group of detection receptors of the innate immune system. These receptors are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production in response to foreign antigens that enter the immune system. It is known that the intestinal epithelial barrier is the largest macro barrier system in the human body, and it is estimated that 60% of the total body immune system lies within the first millimeter of the intestinal lining and is responsible for at least 80% of total body antibody production. Urine IL-6 functions as a marker for the relative activation of the immune system body wide.

Glyphosate

Glyphosate is a synthetic chemical of the organophosphate category. Glyphosate functions as a broad-spectrum antibiotic and weed killer patented by Monsanto as the active ingredient in the herbicide Roundup® that debuted in 1974 in agricultural applications, and later in widespread residential and municipal use. Farmers quickly adopted glyphosate for control of broadleaf weeds and grasses, but application had to be measured and targeted so as not to damage crops. In 1996, Monsanto introduced the glyphosate-resistant “Roundup Ready” crops, enabling farmers to move to spraying entire fields just before seeding as well as throughout the growth and harvest periods without damage to the crop.

By 2007, glyphosate was the most used herbicide in the United States' agricultural sector and the second-most used (after 2,4-D) in home and garden, government and industry, and commercial applications. Today, over four billion pounds of glyphosate is used worldwide. The water-soluble characteristic of glyphosate allows the chemical to move via the root systems of plants with the intracellular water of the primary plant and the resulting fruits or vegetables. In addition, glyphosate can travel in the microecosystem in all phases of the water cycle, contaminating fossil aquafers, ground water runoff, streams, rivers, and oceans, as well as air humidity and rainfall. Studies in the US have demonstrated 75% of air and rainfall samples to be contaminated with glyphosate. Glyphosate kills bacteria, fungi, and plants by blocking the Shikimate enzyme pathway in these organisms. The Shikimate pathway is responsible for producing the carbon-ringed essential amino acids, such as tryptophan, that serve as critical building blocks for

hormones and other proteins in animals and humans. Our group has previously reported that glyphosate can directly damage tight junction proteins in both epithelial (intestinal) and endothelial (vascular) cell barriers.

Zonulin. Zonulin is a pre-haptoglobin and the only endogenous human protein discovered to date that is known to reversibly regulate intestinal and vascular (including renal and blood-brain barrier) permeability by acting as a modulator of the intercellular tight junctions. Zonulin expression can be stimulated by dietary exposure to the gluten breakdown product, gliadin, as well as glyphosate. This exogenous stimulation of zonulin can disrupt tight junction regulation and lead to chronic inflammatory disorders, as well as autoimmune conditions including celiac disease and type 1 diabetes. Both animal studies and human trials using the zonulin synthetic peptide inhibitor AT1001 (now named Larazotide acetate) established that zonulin is integrally involved in the pathogenesis of autoimmune diseases. Zonulin can be used as a biomarker of impaired gut barrier function for several autoimmune, neurodegenerative, and tumoral diseases, and can be a potential therapeutic target for the treatment of these devastating conditions.

Methods

Institutional Review Board approval for the study design, methods of analysis, and informed consent form was obtained previous to solicitation of each subject. Subject selection, randomization, screening, and clinical tracking were performed by a research coordinator that was blinded to active versus placebo status of subjects.

From a pool of 150 healthy, adult volunteers ages 18-55 years, 26 subjects were randomized to two blinded cohorts of thirteen subjects each. Baseline spot urine specimens were collected from all subjects immediately following completion of informed consent and baseline health questionnaire. The treatment group was provided a liquid mineral supplement composed of stabilized aqueous humic substances and conjugate humates, Terrahydrite®, and the placebo group was provided a liquid supplement composed of a color and taste-matched blend of water, herbal tea, and mineral salt. Both placebo and active supplement were provided in identical eight-ounce HDPE plastic bottles marked only with a research code on the bottom. Instructions were provided verbally and in writing to all subjects to consume 5 mL of placebo or aqueous supplement three times daily with meals for two weeks. No dietary changes were supported or reported by any subject participant over the two-week period of study. Repeat spot urine was collected the day following fourteen days of supplementation. Compliance and clinical course were reported via standardized questionnaire.

All baseline and post treatment urine specimens were frozen at -20°C until further analyses. At the end, frozen urine specimens were simultaneously thawed and equivalent aliquots from each specimen were analyzed through enzyme-linked immunosorbent assay (ELISA) for the measurements of urinary TNFα, IL-6, zonulin, and glyphosate.

Data Analysis. Data acquisition, graphing, and statistical analysis were performed with an automated software suite in accordance with published, industry-standard methodology and manufacturer guidelines. The results presented here are the mean values of each subject group. Data is presented as mean values ± standard error. $P<0.05$ was considered statistically significant.

Results

All 26 subjects completed the trial and data collection. No adverse reactions were reported during the two-week study period. All research subjects and the clinical trial coordinator remained blinded until data collection was complete for all subjects.

TNFα. Urine TNFα was assayed to screen for occult acute inflammatory processes in both cohorts. All subjects had urine TNFα values within the normal range for healthy subjects. There was a tendency to reduced levels of urine TNFα in both cohorts (see Figure 2) at the end of study although they did not reach statistical significance.

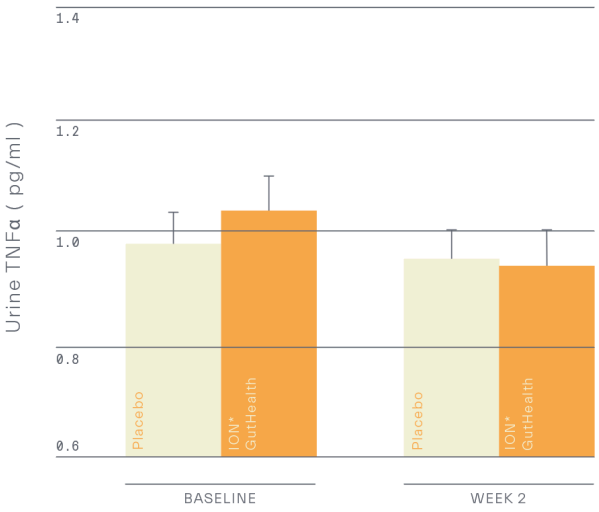

Figure 2. Urine TNFα levels at pre-treatment baseline and at the end of the two-week post-treatment period in both placebo (light bars) and ION*Gut Health treatment (dark bars) groups (n=13, each group).

IL-6. Measurements of urine IL-6 in the placebo group of subjects showed no statistical difference between pre-treatment baseline and two-week post-treatment. In the ION*Gut Health treatment group, ION*Gut Health significantly reduced by 17% the levels of urine IL-6 over the two-week period of the study compared to pre-treatment baseline (Figure 3).

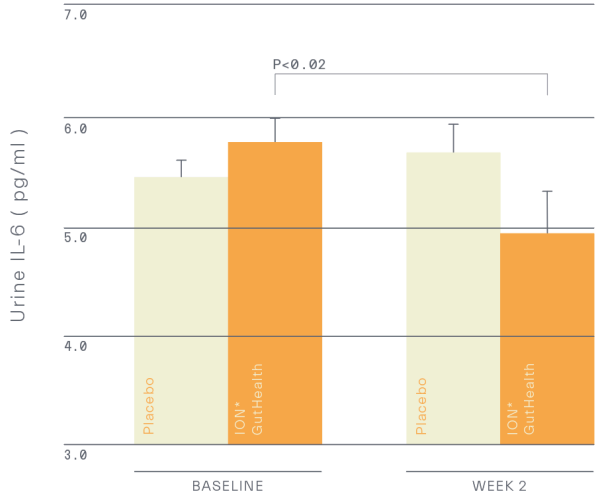

Figure 3. Urine IL-6 levels at pre-treatment baseline and at the end of the two-week post-treatment period in both placebo (light bars) and ION*Gut Health treatment (dark bars) groups (n=13, each group).

Glyphosate. Urine glyphosate was assayed to determine the intestinal absorption and renal clearance of glyphosate present in the food and water consumed by all healthy adult subjects. In the placebo group, there was no difference in levels of urine glyphosate comparing the pre-treatment baseline and the two-week post-treatment. In the ION*Gut Health treatment group, there was a significant reduction of 23% in levels of urine glyphosate over the two-week period of study, compared to pre-treatment baseline. At the end of study (week 2), levels of urine glyphosate were also significantly reduced in ION*Gut Health treated group compared to placebo group of subjects (Figure 4).

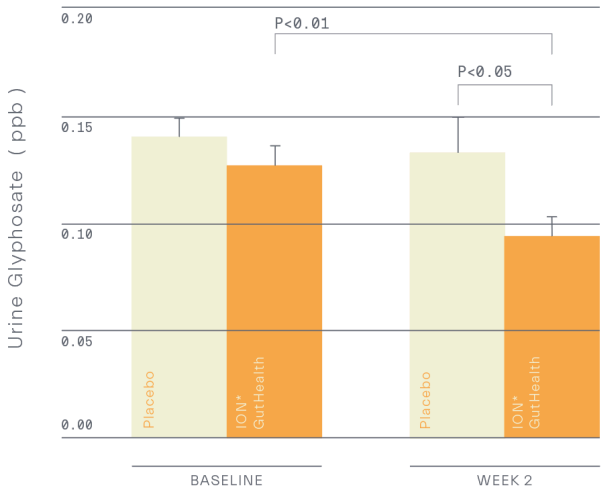

Figure 4. Urine glyphosate levels at pre-treatment baseline and at the end of the two-week post-treatment period in both placebo (light bars) and ION*Gut Health treatment (dark bars) subject groups (ppb = parts per billion; n=13, each group).

Zonulin. Urine zonulin was assayed to determine the cumulative activation of intestinal permeability as produced by dietary glyphosate exposure. In the placebo group, there was no statistical difference in urine zonulin levels comparing the pre-treatment baseline to two-weeks post-treatment. In the ION*Gut Health treatment group, ION*Gut Health significantly reduced, by 12%, levels of urine zonulin over the two-week period of the study compared to pre-treatment baseline (Figure 5).

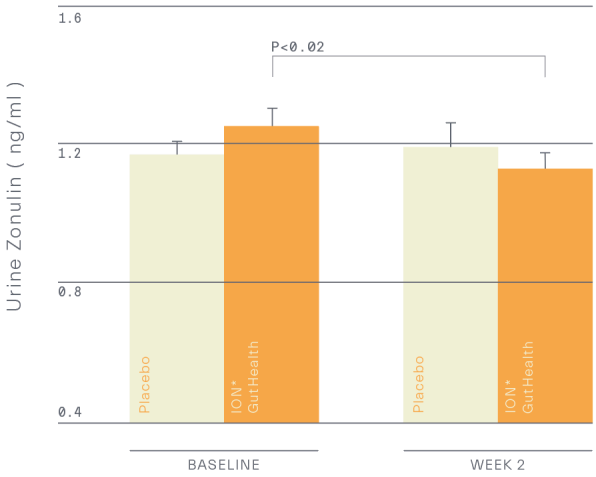

Figure 5. Urine zonulin levels at pre-treatment baseline and at the end of the two-week post-treatment period in both placebo (light bars) and ION*Gut Health treatment (dark bars) groups (n=13, each group).

Discussion

Our research group has previously demonstrated in multiple peer-reviewed in vitro studies the detrimental effects of glyphosate on tight junction permeability through the use of small intestine, colon, and renal vascular cell models. In addition, we have also showed the capacity of the aqueous humic extract supplement – ION*Gut Health® - to support protection and rapid repair of glyphosate injury to these barrier systems. Our current double-blind, placebo-controlled trial evaluated some biologic markers involved with glyphosate absorption, tight junction permeability, and immune system activation in healthy adult subjects. Our results demonstrated in healthy subjects that the two-week supplementation of oral ION*Gut Health in an ambulatory outpatient setting reduced the urinary levels of IL-6, zonulin, and glyphosate, without change in diet or exercise behavior.

Our demonstration of reduced levels of glyphosate in the group of subjects receiving ION*Gut Health over the two-week period of study is likely the result of multiple biologic pathways, including ION*Gut Health-induced upregulation of ZO-1 protein synthesis and tight junction proteins expression at the intestinal barrier, which would improve the regulatory capacity of the barrier system, keeping synthetic and naturally occurring toxins out of the immune and vascular systems. Another important finding of the present study was the reduced levels of urine zonulin in subjects receiving ION*Gut Health for two weeks. Reduced zonulin could be in part due to the upregulation of the enzymatic production of dipeptidyl peptidase 4 (DPP4) by intestinal epithelial cells which would prevent the production of, and accelerate the removal of, zonulin in the pericellular space.

Additionally, we also demonstrated reduced urinary levels of the inflammatory marker IL-6 in the ION*Gut Health-treated group which can reflect a decreased antigen presentation to the innate immune system as a result of diminished levels of glyphosate and reduced intestinal permeability as reflected by the drop in urine zonulin in the same group of individuals.

Our previous in vitro studies have also demonstrated that ION*Gut Health caused a marked reduction in mitochondrial reactive oxygen species (ROS) in healthy cell populations which would have the cumulative effect of reducing oxidative stress on the anti-oxidant pathways at the same time that ION*Gut Health increases glutathione production in intestinal epithelium and hepatocytes.

In conclusion, our current clinical findings demonstrated that ION*Gut Health supplement markedly reduced the urinary levels of the inflammatory marker IL-6, the tight junction modulator zonulin, and the pesticide glyphosate in adult health subjects even in a short two-week supplement course.

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