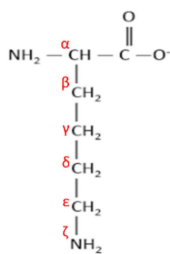


Dietary Supplementation of Terrahydrite® Promotes Lysine Production in Healthy Subjects: A Double-Blind, Placebo-Controlled Clinical Trial

Jiqian Huang MD/PhD, John Gildea PhD, Zach Bush MD

Overview

We performed a double-blind, placebo-controlled clinical trial to study the effects of short term use of the dietary supplement, Terrahydrite®, which has been marketed as RESTORE, and more recently ION^{*} Gut Support. This study followed urine markers in 26 healthy subjects that continued their routine American diet throughout the study. Urine collection was done at baseline and after two weeks of oral use of the active supplement or placebo (usage was 5mL three times daily with meals).



The results of this study continue to reveal many of the functional pathways through which Terrahydrite® supports optimal biology. Among these findings is a marked increase in the essential amino acid (AA), lysine, in the active supplement group. Lysine is designated as an 'essential' amino acid as it cannot be produced by human cells, and therefore it is only through nutritional availability that it is obtained for human cell physiology. Lysine is produced by various bacteria, fungi, and plants through unique enzyme pathways. It serves as a building block to many proteins in the human system that are responsible for regulating genomic response to the environment, protein synthesis and stability, tissue regeneration, and immune system function.

Normal dietary requirements for lysine have been found to be about 8 g per day or 12 mg/kg in adults. Children and infants need more, 44 mg/kg per day for an eleven to twelve-year-old, and 97 mg/kg per day for a three to six-month-old. Lysine is high in foods such as wheat germ and can be found in animal products when their forage is rich in plant varieties that are good sources of lysine. Of meat products, wild game have the highest concentrations of lysine. Fruits and vegetables contain little lysine.

Lysine and Proteinogenesis

Lysine is involved in many biological processes in humans, most importantly proteinogenesis as it serves as one of the 22 amino acid building blocks that compose all proteins. These proteins help produce hormones, immune cells, and enzymes. Lysine also plays an important role in stabilizing three-dimensional protein structures via the positively charged side chain that interacts with water soluble elements, forming hydrogen bonds, salt bridges, and covalent interactions, and also via the long hydrophobic carbon tail close to the backbone, which is buried in the interior of the protein complex [1-4].

Lysine and Epigenetic Regulation

Epigenetic regulation of gene expression is another major function of lysine. In this functional role, lysine acts through histone modification which is responsible for opening up genetic targets for active gene transcription to produce the microRNA (miRNA) that determine cellular responses to, and interaction with, the environment, as well as DNA transcription to messenger RNA (mRNA) in the nucleus of the cell that then moves to the cytoplasm of the cell to allow for translation to protein production [5,6]. There are several types of covalent histone modifications, which commonly involve lysine residues found in the protruding tail of histones. Modifications often include the addition or removal of an acetyl, methyl, ubiquitin, or a sumo protein group [7-10]. The various modifications have downstream effects on gene regulation that influence diverse biological processes such as transcriptional activation/inactivation, chromosome packaging, and DNA damage/repair.

Lysine and Collagen Formation

Collagen is the most abundant protein in the human body. It provides structural support to the extracellular space of connective tissues. Due to its rigidity and resistance to stretching, it is the perfect matrix for skin, tendons, bones, and ligaments. Twenty-eight types of collagen have been discovered and the most common are types I through IV, with type I comprising over 90% of collagen in the human body [11-13]. There is a complex metabolic process that governs the formation of collagen, and lysine is central to the chemical reactions involved. Once integrated into collagen structure, lysine in the protein chain allows for hydroxyl modification and subsequent glycosylation with galactose or glucose intracellularly to allow assembly of the collagen fibril via covalent bonding of lysine and hydroxylysine between collagen molecules extracellularly. The series of reactions decide the type, strength, and stabilization of collagen. Lysine is not only directly involved in the formation of collagen, which is secreted into the extracellular matrix to support tendons and ligaments, but also affects the proper functioning of many other parts of the human body, making it indispensable as far as the overall musculoskeletal health.

ION* Gut Support

ION* Gut Support (IGS) is a dietary supplement that contains a complex family of carbon-based metabolites that are produced by bacteria and fungi in diverse microbiome environments such as pre-agricultural soils. The initial fossilized soil (leornardite-derived) mineral supplement is an alkaline liquid form that carries trace minerals and amino acids at concentrations well below daily dietary intake levels (not adequate to directly raise urine amino acid levels). The functional element of the supplement is the water-stabilized family of carbon-based redox (electrical charge) carrying molecules. We have previously demonstrated and published the function of Terrahydrite® (the active ingredient) to support the repair and protection of the intestinal barrier (tight junctions) in the presence of gluten and/or glyphosate-mediated tight junction injury [17,18].

Methods

Institutional review approval for the study design, methods of analysis, and informed consent form was obtained previously to solicitation of each subject. The 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 randomly selected 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 questionnaires. The treatment group was provided a liquid mineral supplement, ION* Gut Support (IGS), 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 questionnaires.

All baseline and post treatment urine specimens were frozen at -20°C until further analyses. At the end, a frozen urine specimen was simultaneously thawed and equivalent aliquots from each specimen were analyzed through liquid chromatography and mass spectrometry (LC-MS) **by a blinded, third party laboratory.**

Data analysis

Data acquisition, graphing, and statistical analysis were performed with GraphPad Prism 8.3.0 (GraphPad) in accordance with published, industry-standard methodology and manufacturer guidelines. The results were presented as mean values \pm standard error (SE). The $p < 0.05$ was considered statistically significance.

Results

The LC-MS data of all parameters was presented in four randomized groups (Table 1): Baseline groups of Placebo (P_0) and ION⁺ Gut Support (R_0), and two weeks of treatment groups of Placebo (P_2) and ION⁺ Gut Support (R_2). The ROUT method was employed to identify the outlier in each group with 1% Q value. The case number, n , in each group was the final one to be analyzed in successive statistical assay excluding the outliers. The skewness and kurtosis of data sets were documented to show the symmetry and tailed characteristics of data. Data sets were computed for relative likelihood of sampling from *Gaussian* (normal) or lognormal distribution, and *Anderson-Darling* test, *D'Agostino-Pearson* omnibus normality test, *Shapiro-Wilk* normality test, *Kolmogorov-Smirnov* with *Dallal-Wilkinson-Lilliefor* p value were conducted as normality tests. All groups were either normal or lognormal distribution, passed their respective normality tests and were subjected to *One Way ANOVA* assay and follow-up *Turkey's* multiple comparison to control the false discovery rate (Table 1).

Urine Creatinine

The urine creatinine in each sample was used to normalize urine protein clearance among subjects. All urine AA levels were expressed as the ratio of AA to their correspondent creatinine (Table 1). No significant difference of the creatinine was found among P_0 , R_0 , P_2 , and R_2 ($p = 0.7267 > 0.05$, $R^2 = 0.03037$).

L-Asp and L-Asp-derived AA

L-Asp is precursor AA to biosynthesize L-Ile, L-Lys, L-Met, and L-Thr. No significant differences of L-Asp, L-Ile, and L-Met were found among P_0 , R_0 , P_2 , and R_2 and the significant increases of L-Lys ($p = 0.0134 < 0.05$, $R^2 = 0.2376$) and L-Thr ($p = 0.0010 < 0.01$, $R^2 = 0.3318$) in R_2 were demonstrated comparing to its correspondent P_0 , R_0 , and P_2 (Table 1 and Figure 1a).

Basic AA transport system-associated AA

No significant statistical differences of L-Arg ($p = 0.9218 > 0.05$, $R^2 = 0.01166$) and L-Orn ($p = 0.4989 > 0.05$, $R^2 = 0.05829$) were found among P_0 , R_0 , P_2 , and R_2 (Table 1). Comparing to P_0 and R_0 , the lower levels of L-His were shown in both P_2 and R_2 ($p = 0.0011 < 0.01$, $R^2 = 0.3267$) (Table 1). The intergroups difference didn't pass *Turkey's* multiple comparison test.

L-Lys catabolism-associated breakdown product 2-AAA

No significant differences of 2-AAA were demonstrated among P_0 , R_0 , P_2 , and R_2 ($p = 0.0883 > 0.05$, $R^2 = 0.1426$) (Table 1).

The relative abundance of L-Lys to L-Asp and L-Asp-derived AA

Table 1 and Figure 1a show a significant increase of L-Lys in R_2 (R_2 vs P_2 , 85.33 ± 14.03 vs 39.96 ± 9.280 , $p = 0.0136 < 0.05$, $R^2 = 0.2376$). The ratios of both L-Lys / L-Asp (R_2 vs P_2 , 24.47 ± 4.996 vs 14.41 ± 3.086 , $p = 0.0013 < 0.01$, $R^2 = 0.3286$) and L-Lys / L-Ile (R_2 vs P_2 , 7.973 ± 1.286 vs 4.894 ± 1.280 , $p = 0.0156 < 0.05$, $R^2 = 0.2262$) were statistically higher in R_2 (Figure 1b & 1c). The ratios of L-Lys / L-Met (R_2 vs P_2 , $9.495 \pm$

1.158 vs 10.30 ± 3.045 , $p = 0.7172 > 0.05$, $R^2 = 0.03362$) and L-Lys / L-Thr (R_2 vs P_2 , 1.183 ± 0.1728 vs 1.165 ± 0.2644 , $p = 0.5616 > 0.05$, $R^2 = 0.04943$) were not significant differences among P_0 , R_0 , P_2 , and R_2 (Figure 1d & 1e).

The relative abundance of L-Lys to transport system-associated AA

A relatively high abundance of L-Lys to L-Arg and L-Orn was demonstrated (Figure 1f-1g). The ratio of L-Lys / L-Arg (R_2 vs P_2 , 8.080 ± 1.244 vs 3.940 ± 0.9535 , $p = 0.0034 < 0.01$, $R^2 = 0.2929$) was significantly increased in R_2 versus P_2 (Figure 1f). The ratio of L-Lys / L-Orn ($p = 0.0958 > 0.05$, $R^2 = 0.2467$) didn't show significant change among P_0 , R_0 , P_2 , and R_2 (Figure 1g). A relative lower abundance of L-Lys to L-His (0.3160 ± 0.07211 , 0.2250 ± 0.07868 , 0.6264 ± 0.1685 and 0.6125 ± 0.1088 , $p = 0.0294 < 0.05$, $R^2 = 0.1949$) in P_0 , R_0 , P_2 , and R_2 were demonstrated (Figure 1h). The intergroups difference, however, didn't pass *Turkey's* multiple comparison test.

The relative abundance of L-Lys to its catabolic intermediate product 2-AAA

A relatively high abundance of L-Lys to 2-AAA was demonstrated and no significant statistical difference was shown among groups ($p = 0.4681 > 0.05$, $R^2 = 0.03447$) (Figure 1i).

Discussion

An elevated level of urine lysine was seen in human subjects administered with ION* Gut Support during a two-week period. The urine concentrations directly reflect the lysine level in the bloodstream. As an essential amino acid, lysine production in the human subjects is limited to the gut microbiome source. We tracked stability of the nutritional sources of amino acids by measurement of aspartate in the urine of subjects. Aspartate is a precursor amino acid to isoleucine, lysine, methionine, and threonine, and the study showed no significant change in placebo- and IGS-treated groups. This demonstrates that the subjects in both the placebo and IGS-treated groups had a steady and reliable aspartate source from the foods for the biosynthesis of isoleucine, lysine, methionine, and threonine and it is not influenced by placebo or ION* Gut Support.

Previous studies indicate that intestinal bacteria appear to play an important role in the production of essential amino acids by means of de novo biosynthesis in human and animal subjects. In vitro studies have confirmed that human colonic microbiota generate lysine, glutamate, serine, threonine, alanine, isoleucine, valine, and phenylalanine following fermentation with young barley leaf extract [19] via the ruminal bacteria, such as *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii* that are capable of de novo synthesis of amino acids in the presence of physiological concentrations of peptides [20]. In vivo studies have also shown that microbial-derived lysine is absorbed and incorporated into host proteins [21-23].

Based on these studies, and the results of our trial outlined here, we propose that daily use of Terrahydrate® over a two-week period exerts its effect on intestinal bacteria in human subjects to biosynthesize the lysine. Terrahydrate® contains only trace amounts of essential amino acids, including lysine, 100 to 1000 times lower than the daily dietary intake, and therefore are not directly contributing to the lysine levels needed to raise the urinary excretion as seen in this study. The biosynthesis of isoleucine and methionine in this study were not influenced by the placebo or IGS-treated groups, and the relative abundance of lysine compared to isoleucine in IGS-treated subjects suggests a specific upregulation of lysine production from the gut microbiota.

Except aspartate-derived synthesis of lysine (the diaminopimelate (DAP) pathway), lysine also can be biosynthesized via the 2-amino adipate (AAA) pathway. The AAA pathway involves the condensation of α -ketoglutarate and acetyl-CoA via the intermediate 2-amino adipate for the synthesis of lysine. This pathway has been shown to be present in yeast, protists, fungi, *thermus thermophilus*, and *pyrococcus horikoshii* [24-33]. Interestingly the catabolism of lysine is the reverse of AAA pathway. In this study, no

statistically significant change of the levels of 2-aminoadipate was found both in placebo and IGS-treated groups. The elevated ratio of lysine to 2-aminoadipate indicates that increased lysine production is not neutralized by an increase in catabolic metabolism of the lysine.

In the view of an ATP-dependent uptake system, basic amino acids (lysine, arginine, ornithine, and histidine) share the similar amino acid transport system, ABC transporter in prokaryotes [34] and the system b^0+ transporter rBAT/ b^0+ AT (SLC3A1/SLC7A9) in mammal and human [35]. The levels of arginine, ornithine, and histidine were not influenced by IGS-treatment in this study. Higher abundance of lysine verses arginine and ornithine indicates its competitive priority in trans-membrane transport from the gut to the bloodstream in IGS-treated subjects.

In summary, ION* Gut Support promotes the lysine production in healthy subjects, likely by stimulating intestinal microbiota production and intestinal transport of this critical regulatory amino acid. These findings elucidate one of the likely mechanisms by which Terrahydrite® supports tight junction protection and repair following gluten or glyphosate injury as we have previously published, and sheds light on the in vivo function of Terrahydrite® to support intrinsic pathways of protein production, cell repair, and immune function in human and animal subjects.

Perspectives

Dietary supplement, ION* Gut Support, promotes the gut microbiome production of lysine by which it is expected to play an important role in protein production, epigenetic regulation, collagen production, immune system regulation, and a variety of cell processes. Further studies are needed to demonstrate the direct and indirect effects of ION* Gut Support on athletic performance, muscle recovery, joint health, and acute inflammation.

Acknowledgement

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