ioniplex®

unparalleled nutrient absorption™

- **Cellular Health and Energy**
- **Healthy Blood Glucose Levels**
- **Stronger Hair, Skin and Nails**

WHITE PAPER

A clinically validated fulvic ionic mineral complex™ for:

cellular health and energy, healthy blood glucose levels, stronger hair, skin and nails

MINERAL BIOSCIENCES®
Nature Knows Best™

Mineral BioSciences, LLC
4050 S. Sarival Avenue
Goodyear, AZ 85338

OVER 40 YEARS IN BUSINESS

ioniplex®
unparalleled nutrient absorption™

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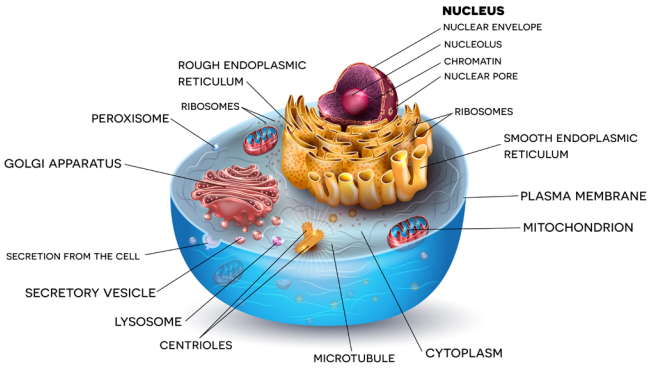
WHITE PAPER

Ioniplex® unparalleled nutrient absorption™ | a fulvic ionic mineral complex™

An Approach to Cellular Health and Energy

Ioniplex translocates rapidly through cellular membranes which makes it highly bioavailable[1]. Inside cells, it is capable of stimulating mitochondrial metabolism by up to 50%[2]. Thus, Ioniplex appears to protect and enhance cellular health in two distinct ways: by decreasing cellular damage from toxins and other contaminants, and by increasing the metabolic rate of mitochondria.

Fig. 1 | Anatomy of a Cell



An Approach to Healthy Blood Glucose

Understanding blood glucose (sugar) begins with knowing how the body reacts to the types of food consumed. Blood sugar does not have to be elevated to diabetic levels in order to cause damage. Clinical studies reveal that even moderately increased blood glucose can lead to cellular damage, which is often irreversible[3]. Even in healthy individuals, blood glucose rises after consuming a meal, especially one that is heavy in carbohydrates. These occurrences are known as post-prandial blood sugar spikes. Ioniplex helps reduce cellular damage caused by this excess glucose to better protect and enhance cells[4].

An Approach to Stronger Nails, Hair and Skin

The beauty and strength of nails, hair and skin depends on our bodies level of cellular health. Ioniplex has been shown to help the body generate Type I Collagen and rehydrate cells to create a more youthful appearance[5]. Ioniplex's ability to stimulate cells also promotes faster growth for hair and nails, while making them stronger[6].

About Ioniplex

Ioniplex is a proprietary fulvic ionic mineral complex, containing upwards of 65 major, minor and trace minerals, which are sourced from unique mineraloid veins located throughout North America.

Millions of years ago, these areas were lush with vegetation, that over time, were compressed into the Earth, forming carbon-based compounds known as "humates" or "humic substances" that are rich in minerals and fulvic acid.

Mineral BioSciences® (MBS) carefully identifies, segregates and recovers this material and transports it to our state of the art facility in Goodyear, Arizona. Here it undergoes a patented extraction process[7] where the mineral rich fulvic material is extracted from the humic.

The resulting product is analytically tested for quality assurance, then stored in bulk to eventu ally be bottled or dehydrated into a water-soluble powder, for use in a broad spectrum of health and personal care products.

Ioniplex & Fulvic Acid

Ioniplex contains naturally occurring fulvic acid, the result is an ionic material full of electrolytes, macro and micro-nutrients for optimal health. Some of the benefits of fulvic acid are: enhanced nutrient absorption, improved immune support and performance as a chelation agent.

Ioniplex & Bioavailability

bi • o • a • vail • a bil • i • ty
/ˌbīōˌəvāləˈbiləbilədē/
noun

the proportion of a substance that enters the circulation when introduced into the body and so is able to have an effect.

The effectiveness of any mineral supplement depends on its bioavailability. For example, Calcium is available in many forms (calcium citrate, calcium carbonate, oyster shell calcium- etc.) each with varying levels of bioavailability, all of which deliver only fractions of the labeled ingre-dient to target organs. Ioniplex is classified as highly bioavailable, thus allowing it to penetrate through cellular membranes and deliver its full impact to metabolic sites within cells.

Description of Test: Bioavailability[1]

Report 697-BIOAV

To study bioavailability, human cells were incubated with Ioniplex to measure:

Binding: The binding of Ioniplex to cells.

Intracellular Delivery: The delivery of Ioniplex within cells, using cell-permeant mineral binding

Intracellular Detoxification: The detoxification properties of Ioniplex within cells using cell-permeant mineral binding dye (Calcein AM). In the first experiment, cells were incubated with either 5% Ioniplex or double-distilled water for 45 minutes, followed by rinsing. Cells were observed with an epiflourescence microscope, in order to visualize and capture the intrinsic fluorescence of Ioniplex.

Results

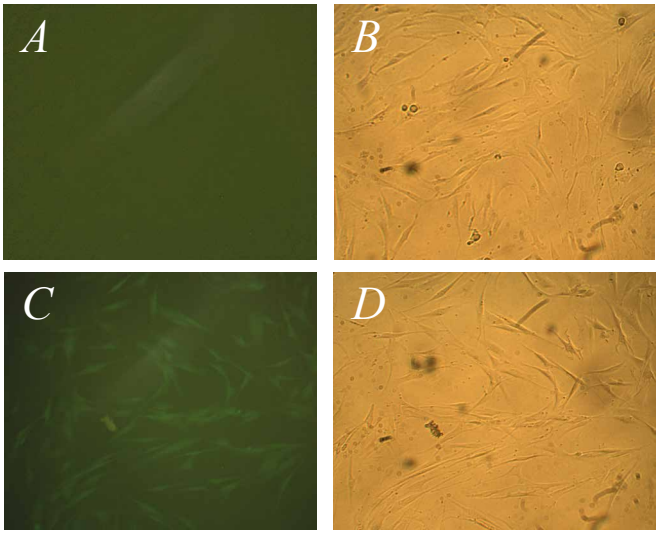
Binding: When cells were rinsed and subjected to fluorescence quantification, it was found that the signal in the Ioniplex treated cultures was over **10 times higher** than the treated controls. Therefore, it can be concluded that Ioniplex associates with cells or cell membranes - a strong indication of its bioavailability.

Intracellular Delivery: The quantification of Mg-Green AM fluorescence, which is proportional to the concentration of intracellular magnesium, showed an overall increase in Ioniplex in treated cell populations.

Intracellular Detoxification: Microscopic observations of the Calcein AM-loaded cells revealed an increase of Calcein fluorescence in the Ioniplex-treated cells. Calcein AM fluorescence increases when the amount of pro-oxidant ions such as cobalt, nickel, iron, and copper decreases. Pro-oxidants induce oxidative stress, therefore, the intracellular increase of Calcein AM in Ioniplex treated cells demonstrates Ioniplex's detoxification / scavenging activity. Together, these results indicate that Ioniplex is a bioavailable vehicle capable of binding cells, delivering ionic mineral payloads inside cells, while detoxifying them by chelating pro-oxidative ions (Fig. 2).

Fig. 2 | Bioavailability Panels

Epiflourescent (left panels) and corresponding bright-field (right panels) images of Calcein AM-loaded control (A,B) and Ioniplex-treated (C,D) human cells. Note the appearance of fluorescent signal in Ioniplex-treated cells, suggestive of calcein de-quenching. Mag x40.



Ioniplex & Mitochondrial Health

mi • to • chon • dri • a
/ˌmīdə ˈkändrēən/
plural noun

Mitochondria are an integral part of a cell and are responsible for metabolizing, or breaking down, carbohydrates and fatty acids in order to generate energy.

Mitochondrial health and metabolism is essential for the healthy condition of the body, since mitochondrial metabolic activity and number decreases with age. Several in vivo studies have shown Ioniplex's ability to promote mitochondrial metabolism. These results are consistent with the notion of Ioniplex having beneficial activity towards maintaining mitochondrial health.

Finally, the gene expression profiling of Ioniplex treated cells revealed an overwhelming stimulation of genes involved in aerobic energy production.

Description of Test: Mitochondrial Metabolism[8]

Report 466A

Ioniplex was added to the popular muscle enhancement product, Ensure® to determine if it would increase metabolic activity in the mitochondria, more so than the standalone product, which acted as the control. To test this theory, cells were incubated either with only Ensure or with Ensure and Ioniplex.

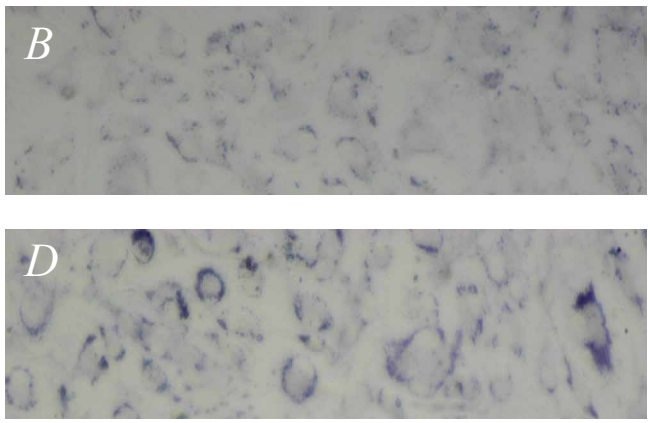
Results

Cells incubated with only Ensure (Fig 3 - Panel B) generated less metabolic activity than cells incubated with Ensure and Ioniplex (Fig. 3 - Panel D). Furthermore, when added, Ioniplex stimulated the expressions of genes that aid in muscle contraction and motility (measured by quantitative PCR).

Fig. 3 | Mitochondrial Panels

Effect of Ensure (top panel) on metabolic activity in mitochondria of human skeletal muscle cells. Cells incubated with Ensure generated less metabolic activity than cells incubated with Ensure and 200 micrograms of Ioniplex (bottom panel).

Note panels B and D show bright field illumination. Magnification x40 with Nikon Eclipse TS100 inverted microscope.



Description of Test: Mitochondrial Metabolism[2]

Report 439

An additional study utilized the colorimetric assay to determine the effects of Ioniplex on mitochondrial metabolism. This test compared the effects of different dosages of Ioniplex with

two leading energy drinks, No Fear® and Full Throttle®.

Results

Among test materials, Ioniplex achieved the greatest stimulation, (over 50% at certain concentrations), and was above or on par with No Fear and Full Throttle.

Finally, in a cell-based DNA microarray study, where the expression of over 20,000 genes was measured in the absence or presence of Ioniplex, it was established that Ioniplex stimulates a whole array of genes involved in mitochondrial metabolism respiration and ATP production[4,9].

Taken together, these results are consistent with the notion of Ioniplex having beneficial activity towards maintaining mitochondrial health.

Ioniplex & Glycation Defense

gly • ca • tion
/ɡlaɪ ˈkeɪʃən/
noun

Glycation (sometimes called non-enzymatic glycation) is a chain of reactions that results in the cross-linkage of sugars with macromolecules, such as proteins and lipids. These cross-linked macromolecules are called Advanced Glycation End-products (AGEs).

In the human body, non-enzymatic glycation often results in alterations of the physiochemical properties of macromolecules, triggering undesirable processes, such as inflammation. In fact, AGEs are markers of physiopathologies such as diabetes and atherosclerosis, and are associated with the aging and photo-aging processes.

Ioniplex may increase metabolic activity by re-routing excess glucose (that would, otherwise, create Advanced Glycation End-products) out of the bloodstream into the cells. The direct result of that action is a decrease in the amount of excess glucose available to mutate into AGEs, which consequently, leads to a decrease in glycation. To confirm, the following studies were conducted.

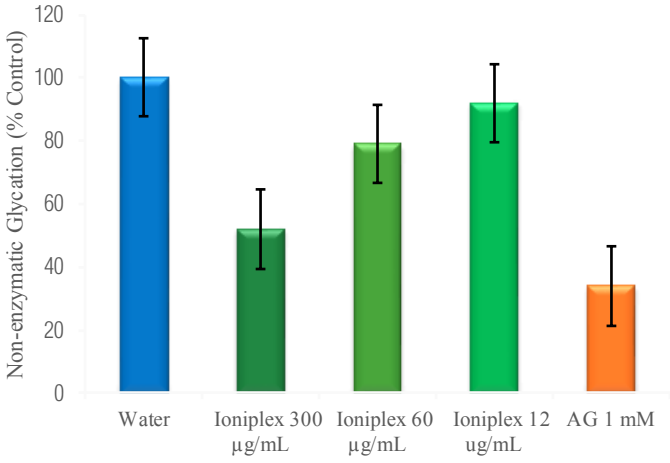
Description of Test: AGE Inhibition[10]
Report MX3

This test measured the inhibition of AGEs using Ioniplex at different doses in comparison to a negative control (water) and a positive control (aminoguanidine). Aminoguanidine is a medicinal inhibitor of non-enzymatic glycation.

Results

Various batches of Ioniplex triggered up to a **50% decrease of AGEs**, which directly correlates to a decrease in the levels of glycation within the body (Fig. 4). These results conclude that Ioniplex has the ability to inhibit AGEs in a dose-dependent manner comparable with aminoguanidine.

Fig. 4 | Inhibition of AGEs



Description of Test: AGE Inhibition[11]
Report AD070618

To validate the inhibition properties of Ioniplex, a second study was performed to determine if the results could be replicated.

A quantitative glycation assay based on the measure of Advanced Glycation end products in proteins was performed. In this protocol, confluent cells are inactivated prior to the labeling experiment. In this model, glycation occurs in mature deposited collagen fibers.

Results

Ioniplex was tested at different dosages against a control (water) and a reference (aminoguanidine). All dosages of Ioniplex showed a decrease in AGEs.

From these results, Ioniplex was patented as an anti-glycation agent to help prevent the occurance of glycation within the body![12].

Ioniplex & Healthy Blood Glucose

blood • glu • cose
/blәd/ /glōōkōs/
noun

Blood sugar, or blood glucose, is the main sugar that the body makes from the food in a person's diet. Glucose is carried through the bloodstream to provide energy to all cells in the body. However, too much glucose in blood can cause serious health issues.

Another effect from the rerouting of excess glucose to the cells and away from the potential for glycation is less glucose present in the bloodstream. Mineral BioSciences is conducting wide ranging human studies to further confirm this process.

Preliminary data suggests Ioniplex positively effects glucose management. Previously an in vitro study was conducted with diabetic mice at the Molecular Medicine Research Institute.

Description of Test: Anti-Diabetic Effects[4]
Report 359

This project aimed to determine the effects of Ioniplex in drinking water on genetically diabetic mice over a period of four weeks. There were two groups that received different dosages of Ioniplex as well as a control group. Water consumption, body weight, blood glucose, glycated hemoglobin and other body chemistry parameters were tested.

Results

Water Consumption: Water consumption between groups was not significantly different.

Body Weight: All groups gained weight during the duration of the experiment. The group drinking 2% Ioniplex gained 6% less weight and the group drinking 10% Ioniplex gained 23% less weight than the control group.

Blood Glucose: All groups registered an increase in glucose levels in the blood, however this increase was reduced by approximatley. 60% in both groups drinking Ioniplex.

Glycated Hemoglobin (HbA1c): The Ioniplex groups registered a 3.5% decrease in the amount of glycated hemoglobin compared to the control.

Among blood chemistry parameters tested, alkaline phosphatase (AP) and albumin/globulin (A/G) ratio were significantly flexed towards non-diabetic levels.

Alkaline Phosphatase (AP): Decreased by approx. 15% by both Ioniplex treated groups. The decrease in AP may be associated with an improvement in diabetic condition, as increased AP has been long known to be a feature of diabetes metillus[13].

Albumin/Globulin (A/G): Increased in a dose dependent manner by 22% (2% Ioniplex) and 35% (10% Ioniplex). An increased ratio of A/G may indicate an attenuation of the prothrombotic environment, characteristic to the physiopathology of diabetes![13].

All other parameters remained unchanged (with-

in 5% of the control), except blood urea nitrogen (BUN), which was moderately increased in the 2% Ioniplex-treated group, and whose significance is unclear, because it was not reproduced in the 10% Ioniplex-treated group.

Taken together, these results demonstrate that administration of Ioniplex in drinking water had multiple beneficial effects on the diabetic animals. These effects included significantly reduced growth of body weight and blood sugar, as well as improved alkaline phosphate and albumin/globulin levels.

Description of Test: Glucose Management[14,15]

Report 697-DIAB / Report 809HbA1c

To determine if the results in the in vivo study conducted on diabetic mice could be replicated in an in vitro study. This test sought the effect of Ioniplex consumption on the blood glucose and glycated hemoglobin of an elderly diabetic patient medicated for hyperglycemia with Metformin.

Hyperglycemia (high blood glucose content) is a hallmark of Type 2 diabetes and a physiopathological effector in this disease. Metformin is one of the most common first-line medications prescribed for lowering hyperglycemia in diabetic patients.

Results

Fasting Blood Glucose: Ioniplex was supplemented at 20 ml/day while maintaining the original Metformin dosage (1 gram/day) which resulted in rapid decrease of fasting blood sugar from an average of 112mg to 92mg.

While maintaining the Ioniplex, Metformin was decreased by 25%. This resulted in the fasting blood glucose sugar level to rise back to the starting average (112mg). However, this indicates that Ioniplex removed the need for 25% of the Metformin medication.

Further improvement of blood glucose levels were observed when the Ioniplex dosage was increased from 20 ml/day to 40 ml/day. This change allowed the Metformin dose to be decreased by another 25%. The result was a blood sugar drop from 112 mg to 105mg. This represents a decrease of about 7% in fasting blood glucose levels and a 50% reduction in medication.

Glycated Hemoglobin: In another clinical case study, HbA1c, also referred to as “glycated hemoglobin”, of the study subject was found to decrease proportionally to the length of the patients’ Ioniplex supplementation.

HbA1c levels were tested multiple times during this 17 week study, at every testing, glycated hemoglobin levels were reduced.

Ioniplex & Hair, Nails & Skin

Description of Test: Collagen Stimulation[5]

Report 400

col • la • gen
/käləjən
noun

Collagen is the main structural protein found in skin and other connective tissue. Type I Collagen is the most abundant collagen found in the human body and supports skin, muscle, bone health, hair and nail growth and maintenance.

Collagen decreases during skin aging and new collagen formation is considered an important activity in wound healing and skin care. This study was meant to detect increases in soluble Type I Collagen in a human dermal fibroblast through Ioniplex application.

Results

Ioniplex was repetitively shown to stimulate Type I Collagen in the fibroblast cells, some results reported as much as **quadruple collagen stimula-**

tion when compared to the control (water). As the level of stimulation depends on the cells used, the average stimulation observed was approximately 30%.

Description of Test: Hair, Nails & Skin[6]
Report 3411BK0609

To assess the effects of Ioniplex at different dosages on nail strength and growth, in addition to hair appearance, luster, thickness and skin condition. The study was conducted over six weeks in a randomized, double-blind, placebo controlled evaluation using three groups of healthy females with at least ten subjects in each group.

Two of the groups received Ioniplex "A" or Ioniplex "B" (Ioniplex at different dosages), and one group received water and flavoring as the Control.

Nail, hair and skin conditions were evaluated visually by an expert evaluator and subjectively by panelists. Nail growth was measured instrumentally using digital photography and subsequent image analysis.

Results

Nail Growth: Percent change as a function of time was observed to be **142% (Ioniplex "A"), 141% (Ioniplex "B")** and 129% (Control)

Nail Strength: Ioniplex "A" affected significant increases in mean nail strength at week six relative to baseline values.

Hair Appearance and Luster: Ioniplex "A" and Ioniplex "B" affected significant increases in the overall appearance of hair. Ioniplex "B" also significantly changed hair luster .

Hair Thickness and Condition: Ioniplex "A" significantly improved hair thickness at week six and hair condition with **14% overall improvement**.

Skin Texture: Ioniplex "A" showed improvement in skin texture at week six compared to the baseline, with an overall improvement of 33%.

Ioniplex & Prebiotics

pre • bi • ot • ic
/prēbīätik/
noun

Prebiotics are nondigestible food ingredients that promote the growth of beneficial microorganisms.

Description of Test: Prebiotic for Skin[16]
Report 912.2

The aim of this project was to investigate the potential of Ioniplex for use as a selective prebiotic for skin commensal bacteria.

Skin and digestive system are two organs in our body, which host a major population of commensal microorganisms. These microorganisms provide crucial support for vital functions of these organs, and their decline due to competition from infectious pathogens contribute to a diverse range of diseases. At the level of the skin, these can range from pruritic, inflammatory conditions such as dermatitis to psoriasis and serious skin infections, like cellulitis.

Ioniplex may be a potential prebiotic due to its track record of beneficial effects on the interaction between plants and their commensal microbiomes. Ioniplex is also a slow-release calcium reservoir, potentially capable of releasing enough calcium on skin to inhibit the growth of pathogenic bacteria such as S. aureus, while promoting the restoration of this ion's natural gradient in the epidermis.

S. aureus and *S. epidermidis* were grown in bacterial medium, after reaching the plateau growth phase microorganisms were diluted 1/1000 in phosphate buffered saline solution (PBS).

Each bacterial strain was seeded either in PBS alone or in PBS supplemented with 0.1%Ioniplex

in K43 medium. After 24h bacterial metabolic activity proportional to bacteria numbers was then assessed. The absorbance of the blue color of formazan proportional to the metabolic activity of the microorganisms was quantified.

Results

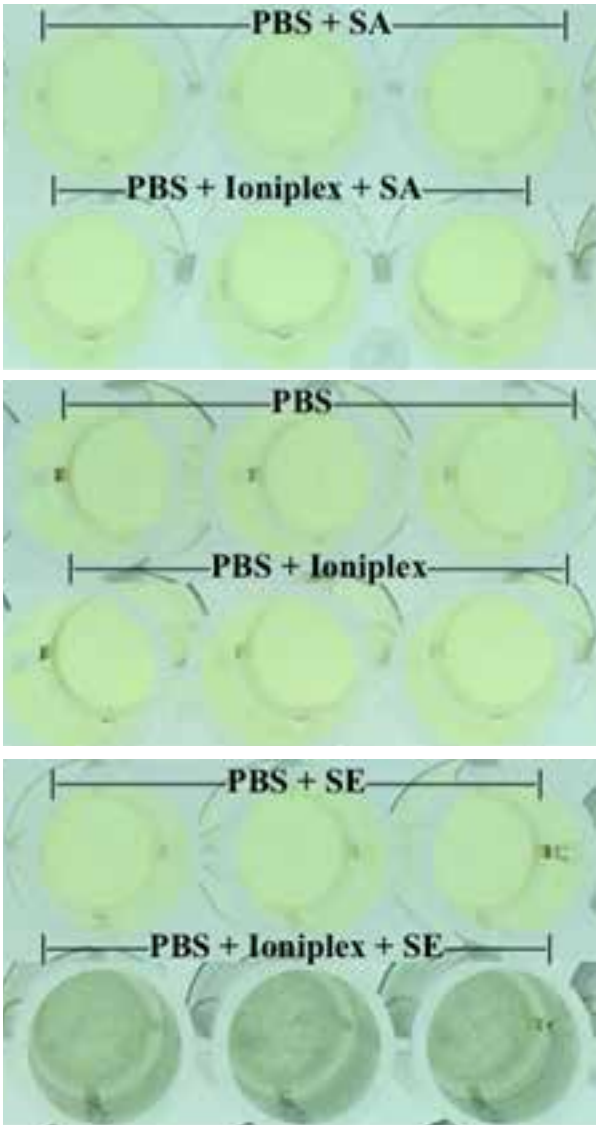


Fig. 5 | Prebiotic Panels

S. epidermidis and pathogenic *S. aureus* bacteria cultured in PBS.

Note darker color, indicative of viability for the commensal (SE) but not the pathogen (SA), when supplemented with Ioniplex.

Neither bacterium grows in PBS alone, providing the technical validation of the experiment.

Figure 5 shows that while the pathogenic bacterium *S. aureus* does not grow in 0.1% Ioniplex. In contrast, the commensal bacterium *S. epidermidis*, shows measurable growth in 0.1% Ioniplex.

None of the microorganisms tested grows in PBS alone, demonstrating the selective prebiotic effect of Ioniplex for the skin-friendly commensal but not pathogenic bacteria.

Ioniplex & CBD

c • b • d
/cbd/
noun

Cannabidiol (CBD) is an active ingredient in cannabis derived from the hemp plant. It may help treat conditions like pain, insomnia, and anxiety.

Description of Test: Epithelial Absorption Potential of Cannabidiol in the Presence or Absence of Ioniplex[17]

Report 1030PAMPA-HPLC

To assess the effects of Ioniplex as a carrier for Cannabidiol or CBD, researchers conducted a study on the absorption rate or bioavailability of CBD through a topical application. Its application through the skin is very popular due to its non-antioxidative, anti-inflammatory, neuroprotective and anti-anxiety properties. Penetration of the stratum corneum or outer layer of the skin (epidermis) was studied.

A Parallel Artificial Membrane Permeability Assay (PAMPA) membrane model showed a high degree of correlation with epithelial permeability. The PAMPA assay with pure CBD (99.9%) as a control was tested along with varied Ioniplex ratios (1:1 and 1:2 ratios of CBD to Ioniplex). The permeated CBD in the assay was analyzed using the Agilent HPLC series 1100. At the 1:2 ratio of CBD to Ioniplex, meaningful and effective CBD epithelial permeation could be detected (Fig 1) compared to CBD alone, wherein no permeation was detected. The permeated CBD assisted by

the Ioniplex was enough to elicit proximal bioactivity.

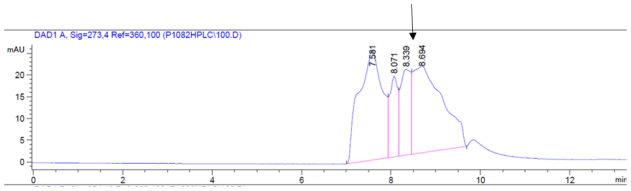


Fig 1| CBD detection in the PAMPA permeation assay using HPLC

Illustrated is the elution profile of CBD permeate as shown by the arrow (CBD peak) from CBD:Ioniplex (1:2) 5000ppm:10,000ppm treatment ratios. Agilent HPLC series 1100 (Agilent Technologies, Palo Alto, CA) station equipped with the Autosampler G1313A, Zorbax SB-C8 reverse phase column, diode array UV/Vis detector was used with Chemstation software and detection was at 273nm (A273nm).

Conclusion

Several clinical studies have shown that Ioniplex is a safe, bioavailable mineral complex with specific properties in: mitochondrial metabolism, glycation defense, maintaining healthy blood and improving how we look and feel. This makes Ioniplex an attractive addition to dietary supplements, functional foods, and personal care products, especially those with a focus on cellular health and/or sugar related issues.

Description of Test:

The aim of this project
was to investigate the potential of Ioniplex and Cannabidiol CBD for use as a daily supplement. Capsules 100 mg of CBD and 200Mg of Ioniplex powder. Also liquid 1oz daily liquid.[18]
Report 10242021P

Administered by Dr Emy Cummins

The study was performed with the participation of 50 patients in two ways:

- 100 mg of CBD and 200 mg of Ioniplex powder Tested with blood lab reports
- Liquid 1oz daily liquid (additive) Tested with blood lab reports

Results

Based on the lab reports from human consumption: oral capsules and liquid.

Human blood trials results show

- Stimulates mitochondrial activity nearly 35%++
- Increases HDL (good) Cholesterol 54%**
- Decreases triglycerides by 66%**
- Decreases Hemoglobin HbA1c by 54%**
- Reduced hs-CRP (systemic inflamation marker)! by 66%**
- Decreased Cortisol by 47%**

Participants reported

- Decreased site pain by 83%**
- Improved sleep by 39%**
- Feeling of increased energy by 44%**
- Feeling of improved mood (anxiety & depression) by 72%**

**Results from In vivo studies involving lab reports from multiple! patients-as well as patients own observations

The lab results have shown great improvement seen with a patient and her kidney health showing a significant increase of GFR. Also observed was a significant change with multiple patient's triglycerides. The decrease in triglycerides can be helped with changes in diet and lifestyle, increased from the out-of-range level.

Notably, this change is challenging to achieve with diet and lifestyle in such a short period of time.

Ionicell CBD can be added to sports drinks and meal replacement drinks. Water soluable allows incorporation in existing products to add a competitive edge to existing products future development.

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All internal reports are available upon request.

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[18] Dr. Emy Cummins (2021). Report 10042021P Based on the lab reports from the human consumption: oral capsules and liquid.

About Mineral BioSciences

Mineral BioSciences, is a division of the Global Organics® Group, and is focused on human and animal wellness. For more than 20 years, we have been producing nutrient-rich mineral complexes for our domestic and international customers. Formulated using patented technology and refined state of the art, USDA certified facilities, our products are Generally Recognized as Safe (GRAS) Affirmed and Kosher Certified.

Subscribing to our corporate motto "Nature Knows Best", and working in concert with our Integrated Life Science Research Center® (ILSRC), Mineral BioSciences continues to identify new and innovative applications of life enhancing mineral formulations for plants, animals and humans .

www.mineralbiosciences.com
www.ioniplex.com
www.protectcells.com

Mineral List

Ioniplex has over 65 plus minerals in its formulation, including:

Guaranteed Composition: Fulvic Acid, Antimony, Sulfur, Barium, Boron, Calcium, Chromium, Cobalt, Iodine, Lanthanum, Magnesium, Manganese, Molybdenum, Neodymium, Phosphorus, Potassium, Rubidium, Selenium, Silicon, Silver, Sodium, Vanadium, Zinc, and Zirconium.

Additional Composition: Carbon (Total Organic), Iron, Copper, Germanium, Gold, Platinum, Sulfur, Fluoride, Niobium, Iridium, Strontium, Titanium, Palladium, Tungsten, Tin, Rhenium, Nickel, Lithium, Gallium, Yttrium, Bismuth, Hafnium, Cadmium, Thorium, Cerium, Tellurium, Beryllium, Samarium, Dysprosium, Erbium, Indium, Scandium, Ruthenium, Tantalum, Rhodium, Thulium, Thallium, Holmium, Ytterbium, Terbium, Lutetium, Gadolinium, Europium, and Praseodymium.

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Patent & Publication

US Patent No. 8,709,497

Title: Mineral, Cosmetic, Pharmaceutical, and Agriculture Compositions and Methods for Producing the Same

This invention pertains to a method for producing compositions including an unusually large number of naturally occurring minerals and compositions that include a significant number of mineral elements and that facilitate delivery of the minerals into the body of the human and animal that pertain to cosmetic, pharmaceutical, agricultural, nutraceuticals.

US Patent No. 8,927,031

Title: Anti-Glycation Methods and Compositions

This invention relates to compositions used to treat or prevent glycation events and related pathologies in humans and animals. More particularly, the invention relates to compositions having anti-glycation activities.

US Patent No. 11103001

Title: Methods and Compositions for Modulating Muscle and Bone Loss

This invention relates to methods and compositions used to slow, reduce, or prevent age-related muscle degeneration and related pathologies in humans and animals.

International Journal for
Vitamin and Nutrition Research

Int. J. Vitam. Nutr. Res., 81 (1), 2011, 34–42

Original Communication

Anti-Diabetic Activity of a Mineraloid Isolate, in vitro and in Genetically Diabetic Mice

Joel Deneau[1], Taufeeq Ahmed[2], Roger Blotsky[3], and Krzysztof Bojanowski[1]

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Received: July 2, 2010; Accepted: November 5, 2010

Abstract: Type II diabetes is a metabolic disease mediated through multiple molecular pathways. Here, we report anti-diabetic effect of a standardized isolate from a fossil material – a mineraloid leonardite – in in vitro tests and in genetically diabetic mice. The mineraloid isolate stimulated mitochondrial metabolism in human fibroblasts and this stimulation correlated with enhanced expression of genes coding for mitochondrial proteins such as ATP synthases and ribosomal protein precursors, as measured by DNA microarrays. In the diabetic animal model, consumption of the Totala isolate resulted in decreased weight gain, blood glucose, and glycated hemoglobin. To our best knowledge, this is the first description ever of a fossil material having anti-diabetic activity in pre-clinical models.

Key words: Leonardite, mineraloid, diabetes, non-enzymatic glycation, mitochondrial metabolism, genetically diabetic mice, Totala

Introduction

Type II diabetes is a metabolic disease characterized by high blood glucose, due to insulin resistance. High glucose triggers pathologic non-enzymatic glycation (NEG) of macromolecules within and beyond the circulatory system, resulting in the formation of advanced glycation endproducts (AGEs). These adducts have multiple biochemical and physico-chemical implications in the body ranging from triggering inflammatory responses through the RAGE receptors, to blocking capillary circulation due to the stiffening of erythrocytes [1]. Removal of AGEs, repair or replacement of the glycated macromolecules, and regeneration of the affected tissues requires extra energy. However, type II diabetes is a disease where mitochondria, the cellular powerhouses, have been found to be dysfunctional [2] and their metabolism impaired [3]. Therefore, inhibition of non-enzymatic glycation and simultaneous stimulation of mitochondrial metabolism are the desirable components of any strategy addressing the diabetic condition.

Natural products are an obvious source to search for potential anti-diabetic regimens. Several plant extracts have been reported to exhibit anti-diabetic activity [4–5]. Here, for the first time, we describe anti-diabetic activities of an isolate – named Totala – obtained not from a contemporary plant, but from a fossil botanical material – leonardite – a mineraloid naturally occurring as an oxidation product of lignite,

a type of coal, dating back to the Tertiary period (65.5 to 1.8 million years ago).

Materials and methods

Materials

The mineraloid isolate, provided by Global Organics (Goodyear, AZ), was obtained by a mild acidic extraction process, as described elsewhere [6] and was always assayed as an aqueous solution. The methods used for the determination of its components are provided in Table II. Total solids and total minerals (ashes) were determined according to standard AOAC methods. Inductive Coupled Plasma Atomic Mass Spectrometry (ICP-MS) analysis was performed with the LECO 2000 combustion analyzer. Neonatal human dermal fibroblasts were from ATCC (Manassas, VA, cat. # PCS-201-010) and adult human dermal fibroblasts were from Cell Applications (San Diego, CA, cat. # 106-05 A).

All chemicals were from Sigma (St. Louis, MO), except AAPH (Wako Chemicals, Richmond, VA).

Mitochondrial activity assay

The mitochondrial activity was measured using the standard thiazolyl blue (MTT) assay as described in [7] with minor modifications. Briefly, normal human dermal fibroblasts (HDF) were incubated with test materials for 3 days in a 96-well plate in DMEM/5 % calf serum, then MTT was added at final concentration of 250 µg/mL. After 2.5 hours, the cell culture medium was removed and the intracellular formazan resulting from the cleavage of the tetrazolium ring by mitochondrial dehydrogenases was extracted with isopropanol and measured with BioRad (Hercules, CA) UV-3550 microplate spectrophotometer at a wavelength of 570 nm with background subtraction at 655 nm.

Table I: Mineral Composition of the Totala isolate as determined by Inductive Coupled Plasma Atomic Absorption (% of total solids)

Element	% w:w
Calcium	5.4
Potassium	1.5
Sodium	1.6
Magnesium	1.1
Aluminum	1.6
Sulfur	3.1
Iron	0.8
Other 65 trace minerals	0.4
TOTAL	15.6

Table II: Organic Composition of the Totala isolate (% of total solids)

Component	% w:w	Method of analysis
Organic acids other than amino acids	77.3	AOAC991.43, calculations
Amino acids	3.1	ICP (LECO 2000)
Saturated fatty acids	0.8	USFDA
Mono-unsaturated fatty acids	1.0	USFDA
Poly-unsaturated fatty acids	0.8	USFDA
Omega fatty acids	1.5	USFDA
Total	84.5	

Non-enzymatic glycation assay

Non-enzymatic glycation (NEG) quantification emulated the method by Suzuki and colleagues [8], with modifications. Briefly, test materials were added to black 96-well plates containing 10 mg/mL bovine serum albumin (BSA, Sigma cat.# A3803) in the absence or presence of 0.5 M glucose (Sigma cat. # G8270). Plates were sealed and the initial non-tryptophan fluorescence was recorded at Ex/Em wavelengths 409 nm/460 nm with microplate fluorometer Cytofluor 4000 (Applied BioSystems, Carlsbad, CA). Aminoguanidine (1 mM, Sigma cat.# 396494) was used as the positive control and sodium azide (0.025 %) was added to prevent biological contamination. After 10–14 days of incubation at 37°C fluorescence was red again and the signal increase between the first and the last day of the incubation was calculated for each experimental point. Finally, the difference between the signal increase for each sample incubated in the presence and absence of glucose was obtained and standardized to water control.

The effect of the mineraloid isolate on non-enzymatic glycation was also measured using extracellular matrix of normal human dermal fibroblasts (HDF), by dot blot. Briefly, HDF were incubated with 20 µg/mL ascorbic acid to enhance the synthesis of collagens for 96 hours; afterwards cell cultures were terminated and cells were lysed by rounds of freezing/thawing. The resulting proteinaceous scaffolding was incubated with

test materials for 15 days in the presence of 20 mg/mL glucose at 37°C/5 % CO_2. At the end of the incubation, proteins were extracted and transferred onto nitrocellulose membranes (Hybond ECL, GE Healthcare, Piscataway, NJ) using Milliblot dot blot device (Millipore, Bedford, MA). Non-specific binding was preempted by overnight incubation with PBS/0.05 % Tween 20/fat-free milk. The membranes were then extensively washed and incubated with a monoclonal anti-AGE antibody (Euromedex, Souffelweyersheim, France, cat. # KH001), followed by peroxidase-linked secondary antibody (Sigma, cat. # A9044) and the chemoluminescent ECL signal proportional to the peroxidase activity was quantified using Fujifilm LAS 3000 scanner. Data were processed with Multi Gauge 3.0 software. Again, aminoguanidine was used as positive control.

DNA microarrays

Adult human fibroblasts were incubated with 2 samples of 30 mg/mL aqueous mineraloid solution at a final dilution of 150 µg/mL in DMEM (high glucose)/5 % FCS for 48 hours; afterward RNA was extracted with RNeasy Qiagen kit. The quality of extracted RNA was assayed twice by electrophoresis (after extraction and before micro array analysis). Samples were hybridized in technical duplicates using human OneArray platform from Phalanx Biotech (Palo Alto, CA). Array data were normalized using the Quantile normalization method (http://bmbolstad.com/stuff/qnorm.pdf). The Excel file yielding information on over 30,000 probes was then further processed to eliminate differences with high p values ($p > 0.1$) and low fold change (< 2). Correction for multiple hypothesis testing was performed using the Benjamini-Hochberg method. Furthermore, all genes described as "uncategorized" and "putative" were excluded from the final analysis. Finally, Array Studio V2.5 (Omicsoft) software was used to identify functional categories affected by the test materials.

Diabetic mouse model

Thirty genetically diabetic male mice (6 weeks old, BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J, stock # 000642, HOM1 – homozygous for the leptin receptor *Lepr,* Jackson Lab) were divided in 3 groups of 10 animals and were given either water (Group 1), water supplemented with the test material at 3 mg/mL (Group 2), or at 0.6 mg/mL (Group 3), for the duration of the experiment (31 days). Animals were kept at 5 per cage and water consumption was monitored. Body weight and blood sugar (non-fasted) were determined on the first and last day of the experiment. Glycated hemoglobin (HbA1C) and blood chemistry markers were measured on the last day of the experiment, using Cobas c-501 clinical laboratory analyzer (Roche Diagnostics, Indianapolis, IN). Glycated hemoglobin was determined using the turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood method [9–10].

The animal facility used was licensed and complied with the U.S. Public Health Service Policy on the Human Care and Use of Laboratory Animals and all of the applicable provisions of the Animal Welfare Act, and other federal statutes and regulations relating to animals, and was guided by the U.S. Government Principles for the Utilization and Care of Vertebrate Animals in Testing, Research and Training (USDA). The animal experimentation protocol for this project was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) – protocol number 07–007.

Statistical Analysis

For all *in vitro* experiments except DNA microarray, results are reported as the mean±standard error of the mean. Statistical significance of differences between the control group and each experimental group was examined using paired Student *t*-test, and p values of < 0.05 were considered significant. For the animal experiments, non-parametric Wilcoxon Rank Sum Test was also used and it corroborated the parametric *t*-test.

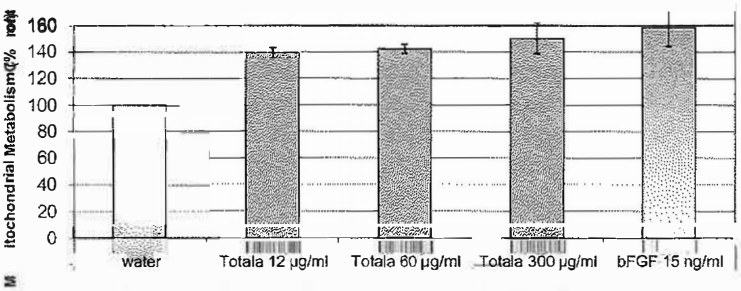

Figure 1: Dose-dependent stimulation of the mitochondrial metabolism in human dermal fibroblasts by Totala mineraloid isolate, as compared with basic fibroblast growth factor (bFGF), assessed by the MTT method ($p < 0.05$ vs. water control for all experimental points). Correct spacing of units (e. g. 300_ug/ml) and ml to mL.

Results

As shown in Tables I and II, the studied mineraloid, Totala, is composed of both organic components (84.5 % w:w) and minerals (15.6 % w:w) with prevalent non-amino and amino acids in the organic group and calcium and sulfur in the inorganic group. Of interest is also the presence of microelements, such as omega fatty acids (1.5 % w:w total). The total solids and ash contents were 91.9 % and 27.8 %, respectively (w:w).

We tested the above-described composition for biological activities relevant to diabetes. One such activity is stimulation of mitochondrial metabolism, as the reduced mitochondrial oxidative capacity has been linked to insulin resistance [11]. We found that Totala strongly stimulates the activity of mitochondrial reductases – key enzymes in the mitochondrial respiratory chain – at the microgram-per-milliliter range of concentration (Figure 1).

We further tested the effect of Totala on non-enzymatic glycation (NEG). NEG, leading to the formation of advanced glycation endproducts (AGEs), is one of the effectors in diabetic progression and complications, such as neuro- and retinopathy (see reference 1 for a recent review).

The mineraloid isolate was found to inhibit NEG using two different methods. The first method depended on the quantification of the increase of the non-tryptophan fluorescence of bovine serum albumin (BSA), due to the conformational change induced by the reaction with glucose. As illustrated in Figure 2, Totala inhibited this reaction by up to 48 % in a dose-dependent manner. Using this technique, the anti-glycation activity of Totala isolate was then compared to other compounds reported to have anti-glycation activity – benzoylthiamine, histidine, pyridoxine (vitamin B6), and lipoic acid (all at 200 µg/mL). It was found that while Totala and lipoic acid had a comparable effect (26 % and 24 % inhibition, respectively), other tested materials inhibited NEG by 15 % or less (results not shown).

The second method relied on measuring the effect of Totala on the glycation of extracellular matrix proteins deposited by human dermal fibroblasts. These proteins (mostly collagen) were incubated in the presence of different concentrations of Totala ranging from 0.1 to 250 µg/mL, then blotted on a nitrocellulose membrane, after which AGEs were detected using specific monoclonal antibodies. This technique demonstrated a decrease of NEG in the presence of Totala by about 16 % across all concentrations tested (Table III), confirming thus the results obtained with the first method (Figure 2). Aminoguanidine, a strong inhibitor of NEG reaction induced by diabetic conditions [12], was used as positive

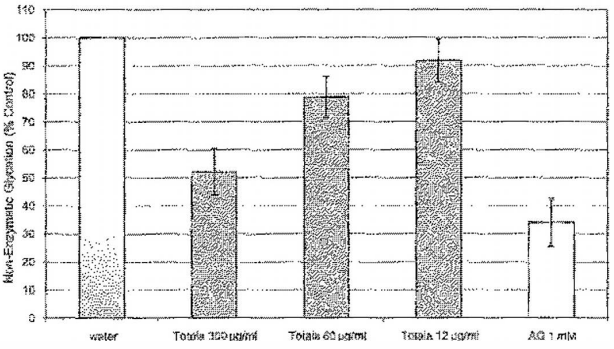

Figure 2: Dose-dependent inhibition of non-enzymatic glycation in the glucose-albumin model by Totala mineraloid isolate, as compared with aminoguanidine (AG), assessed by non-tryptophan fluorescence ($p < 0.05$ vs. water control for all experimental points). Correct spacing of units (e.g. 300 µg/ml) and ml to mL.

control in both experiments and its inhibitory effect was found to be 66 % when using BSA, and 38 % when using the extracellular matrix as the glycation substrate.

In order to identify the molecular mechanisms underlying the effects of Totala, we analyzed its modulation of gene expression, using human fibroblasts. Interestingly, we found that Totala enhanced the expression of a host of genes involved in mitochondrial respiration and ATP synthesis, which is in agreement with its metabolism-stimulatory activity identified by the MTT assay. Furthermore, the increase of expression of multiple mitoribosomal proteins could be detected and Cu-Zn superoxide dismutase (SOD1) was found to be upregulated by a factor of 2.1 (Table IV).

Given its anti-diabetic *in vitro* activity profile, Totala was further tested in a genetically-diabetic db/db mouse model (Table V). During the 31 days of oral administration of the mineraloid in drinking water at 0, 0.6 mg/mL and 3 mg/mL, the liquid intake between the 3 groups was not significantly different ($p > 0.05$). All groups gained weight during the duration of the experiment. The group drinking 0.6 mg/mL Totala gained 6 % less weight, and the group drinking 3 mg/mL Totala gained 23 % less weight than the control

Table III: Effect of the Totala isolate on the non-enzymatic glycation of extracellular matrix deposited by human dermal fibroblasts

Test Material	Advanced Glycation Endproducts (% Control)	SEM
Water	100	3.4
Totala 25 µg/mL	89	12.6
Totala 250 µg/mL	79	6.2
Aminoguanidine 1mM	62	13.1

group. The 3 mg/mL Totala group's weight gain reduction was statistically significant ($p < 0.05$).

All groups registered an increase of glucose level in the blood; however this increase was about 60 % smaller in both groups drinking Totala-supplemented water.

The Totala-drinking groups registered a 3.5 % decrease in the amount of glycated hemoglobin (HbA1C). This slight decrease was not statistically significant ($p > 0.05$). Longer treatment could possibly result in bigger differences.

Among blood chemistry parameters tested, alkaline phosphatase (AP) and albumin/globulin (A/G) ratio were significantly ($p < 0.05$) flexed towards non-diabetic levels: AP was decreased by about 15 % by both Totala groups, while A/G ratio was increased in a dose-dependent manner by 22 % (low Totala group) and 35 % (high Totala group). All other parameters tested remained unchanged (within 5 % of the control), except blood urea nitrogen (BUN), which was moderately increased in the low Totala group, and whose significance

Table IV: Upregulation of mitochondrial proteins

Fold Change vs. Control	P value	Gene
2.6	3.52E-09	ATP synthase delta chain, mitochondrial precursor [SWISSPROT;Acc:P30049]
3.4	4.26E-12	ATP synthase e chain, mitochondrial [SWISSPROT;Acc:P56385]
3.2	2.14E-07	ATP synthase f chain, mitochondrial [SWISSPROT;Acc:P56134]
2.8	9.46E-14	ATP synthase lipid-binding protein, mitochondrial precursor [SWISSPROT;Acc:P48201]
2.3	9.69E-10	ATP synthase O subunit, mitochondrial precursor [SWISSPROT;Acc:P48047]
3.1	5.06E-09	ATPase inhibitor, mitochondrial precursor [SWISSPROT;Acc:Q9UII2]
2.0	7.57E-05	Ubiquinol-cytochrome c reductase complex 11 kDa protein, mitochondrial precursor [SWISSPROT;Acc:P07919]
2.2	1.43E-06	Ubiquinol-cytochrome c reductase complex 11 kDa protein, mitochondrial precursor [SWISSPROT;Acc:P07919]
3.5	1.97E-11	Ubiquinol-cytochrome c reductase complex 6.4 kDa protein [SWISSPROT;Acc:O14957]
2.8	4.42E-13	Ubiquinol-cytochrome c reductase complex ubiquinone-binding protein QP-C [SWISSPROT;Acc:O14949]
2.3	2.05E-12	Cytochrome b-245 light chain (cyt. b558 subunit alpha) (Superoxide-generating NADPH oxidase light chain) [SWISSPROT;Acc:P13498]
2.8	5.86E-15	Cytochrome c oxidase polypeptide VIc precursor [SWISSPROT;Acc:P09669]
3.0	2.08E-08	Cytochrome c oxidase polypeptide VIIa-heart, mitochondrial precursor [SWISSPROT;Acc:P24310]
3.1	0.0477	Cytochrome c oxidase polypeptide VIIa-liver/heart, mitochondrial precursor [SWISSPROT;Acc:P14406]
2.6	1.89E-06	Cytochrome c oxidase polypeptide VIIb, mitochondrial precursor [SWISSPROT;Acc:P24311]
3.0	2.25E-12	Cytochrome c oxidase polypeptide VIIb, mitochondrial precursor [SWISSPROT;Acc:P24311]
3.4	0.0386	Cytochrome c oxidase polypeptide VIII-liver/heart, mitochondrial precursor [SWISSPROT;Acc:P10176]
2.4	2.60E-08	Cytochrome c oxidase subunit 5A, mitochondrial precursor [SWISSPROT;Acc:P20674]
2.6	9.05E-13	Cytochrome c oxidase subunit 5B, mitochondrial precursor [SWISSPROT;Acc:P10606]
2.1	1.16E-05	Cytochrome c oxidase subunit 7C, mitochondrial precursor [SWISSPROT;Acc:P15954]
2.4	7.00E-07	Cytochrome c oxidase subunit VIb isoform 1 [SWISSPROT;Acc:P14854]
4.0	5.61E-12	Cytochrome c oxidase subunit VIb isoform 1 [SWISSPROT;Acc:P14854]
2.5	0.0033	Cytochrome P450 2C18 (EC 1.14.14.1) (CYPIIC18) [SWISSPROT;Acc:P33260]
2.0	0.0379	Cytochrome P450 2C19 [SWISSPROT;Acc:P33261]
6.2	0.0261	Cytochrome P450 7A1 (Cholesterol 7-alpha-monooxygenase) (CYPVII) (SWISSPROT;Acc:P22680]
4.1	0.0721	cytochrome P450, family 4, subfamily F, polypeptide 22 [RefSeq_peptide;Acc:NP_775754]
2.2	6.35E-10	28S ribosomal protein S11, mitochondrial precursor (S11mt) (MRP-S11)[SWISSPROT;Acc:P82912]
2.3	0.0122	28S ribosomal protein S12, mitochondrial precursor (S12mt) (MRP-S12) [SWISSPROT;Acc:O15235]
2.6	1.50E-06	28S ribosomal protein S15, mitochondrial precursor (S15mt) (MRP-S15) [SWISSPROT;Acc:P82914]
2.7	1.00E-08	28S ribosomal protein S15, mitochondrial precursor (S15mt) (MRP-S15) [SWISSPROT;Acc:P82914]

Table IV: Continued

Fold Change vs. Control	P value	Gene
2.2	0.006	28S ribosomal protein S16, mitochondrial precursor (S16mt) (MRP-S16) [SWISSPROT;Acc:Q9Y3D3]
2.2	3.56E-07	28S ribosomal protein S17, mitochondrial precursor (S17mt) (MRP-S17) [SWISSPROT;Acc:Q9Y2R5]
3.6	7.96E-10	28S ribosomal protein S18c, mitochondrial precursor (MRP-S18-c) [SWISSPROT;Acc:Q9Y3D5]
2.8	5.67E-12	28S ribosomal protein S24, mitochondrial precursor (S24mt) (MRP-S24) [SWISSPROT;Acc:Q96EL2]
2.2	5.01E-09	3'-5' exoribonuclease CSL4 homolog (EC 3.1.13.-) (Exosome component 1) [SWISSPROT;Acc:Q9Y3B2]
3.6	5.40E-14	39S ribosomal protein 54, mitochondrial precursor (L54mt) (MRP-L54) [SWISSPROT;Acc:Q6P161]
2.2	1.15E-07	39S ribosomal protein L11, mitochondrial precursor (L11mt) (MRP-L11) [SWISSPROT;Acc:Q9Y3B7]
2.0	2.24E-07	39S ribosomal protein L12, mitochondrial precursor (L12mt) (MRP-L12) (5c5-2) [SWISSPROT;Acc:P52815]
2.1	3.51E-08	39S ribosomal protein L12, mitochondrial precursor (L12mt) (MRP-L12) (5c5-2) [SWISSPROT;Acc:P52815]
2.1	4.78E-10	39S ribosomal protein L13, mitochondrial (L13mt) (MRP-L13) [SWISSPROT;Acc:Q9BYD1]
2.3	1.10E-10	39S ribosomal protein L14, mitochondrial precursor (L14mt) (MRP-L14) (MRP-L32) [SWISSPROT;Acc:Q6P1L8]
2.9	2.57E-10	39S ribosomal protein L14, mitochondrial precursor (L14mt) (MRP-L14) (MRP-L32) [SWISSPROT;Acc:Q6P1L8]
3.2	1.18E-11	39S ribosomal protein L21, mitochondrial precursor (L21mt) (MRP-L21) [SWISSPROT;Acc:Q7Z2W9]
2.6	4.61E-08	39S ribosomal protein L22, mitochondrial precursor (L22mt) (MRP-L22) [SWISSPROT;Acc:Q9NWU5]
2.6	2.38E-10	39S ribosomal protein L24, mitochondrial precursor (L24mt) (MRP-L24) [SWISSPROT;Acc:Q96A35]
2.6	4.33E-09	39S ribosomal protein L36, mitochondrial precursor (L36mt) (MRP-L36) [SWISSPROT;Acc:Q9P0J6]
2.5	2.53E-11	39S ribosomal protein L40, mitochondrial precursor (L40mt) (MRP-40) [SWISSPROT;Acc:Q9NQ50]
3.5	2.42E-10	39S ribosomal protein L41, mitochondrial precursor (L41mt) (MRP-L41) [SWISSPROT;Acc:Q81X
2.1	5.97E-05	39S ribosomal protein L46, mitochondrial precursor (L46mt) (MRP-L46) [SWISSPROT;Acc:Q9H2W6]
3.0	5.47E-11	39S ribosomal protein L51, mitochondrial precursor (L51mt) (MRP-L51) [SWISSPROT;Acc:Q4U2R6]
2.8	3.24E-08	39S ribosomal protein L53, mitochondrial precursor (L53mt) (MRP-L53). [SWISSPROT;Acc:Q96EL3]
2.2	5.34E-09	39S ribosomal protein L55, mitochondrial precursor (L55mt) (MRP-L55) [SWISSPROT;Acc:Q7Z7F7]
2.1	3.38E-08	Superoxide dismutase [Cu-Zn] [SWISSPROT;Acc:P00441]

is unclear, because it was not reproduced in the high Totala group.

Discussion

Type II diabetes is a metabolic disease with complex etiology. Here, we report a surprising discovery that a fossil mineraloid isolate can improve some of the pathophysiological markers of this disease – impaired mitochondrial metabolism and non-enzymatic protein glycation – *in vitro*, translating into improved blood chemistry and body weight parameters in genetically diabetic mice.

Mitochondrial metabolism determines cellular energy level, which in turn controls tissue regenerative

potential. Mitochondrial metabolic activity has also been directly implicated in the regulation of blood glucose homeostasis and thus in diabetes [13]. In fact, reduced mitochondrial oxidative capacity has been shown to increase the accumulation of intramuscular fat, causing insulin resistance with aging [11]. Totala was found to stimulate mitochondrial metabolism by two distinct methods. The first, colorimetric method quantifies the conversion of the soluble yellow MTT dye to the insoluble purple formazan by mitochondrial dehydrogenases, such as succinate dehydrogenase. This conversion is proportional to the metabolic activity in the mitochondrium [14] and the mineraloid isolate stimulated it not only in the usual model of neonatal cells, but also in adult human fibroblasts, which are usually less responsive to the external stimuli.

The second method consisted in quantifying gene expression by DNA microarray technology and mining the resulting database for components of the mitochondrial respiratory chain differentially expressed in the presence and absence of Totala. As illustrated in Figure 1 and Table IV, the results of both methods converge, demonstrating the mitochondrial activity stimulation potential for this mineraloid isolate, *in vitro*. Interestingly, besides the genes directly involved in mitochondrial respiration, we found an upregulation of expression for mitoribosomal proteins. Mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and are transported to the mitochondrion to help in protein synthesis [15]. The upregulation of the expression of these genes further points to the overall metabolism enhancement by the isolate. The underlying mechanism needs further investigation. High content of magnesium in Totala may be one of the effectors, as this mineral is known to be a key cofactor for mitochondrial respiration reactions (see reference 16 for recent review). Of note also is the upregulation of copper/zinc-dependent superoxide dismutase (SOD1) gene expression by Totala, indicating its potential as mitochondrium-protective anti-oxidant [17] and adding another element to its overall anti-diabetic mechanism of action.

One of the emerging paradigms in diabetes is the pro-inflammatory process called non-enzymatic glycation (NEG) caused by excessive blood glucose and leading to the accumulation of advanced glycation endproducts (AGEs). NEG is a physiopathological effector involved in diabetic complications, and inhibitors of non-enzymatic glycation such as pyridoxamine have been demonstrated to inhibit the development of retinopathy and neuropathy in the streptozotocin-induced diabetic rat [18–19]. Therefore treatment with AGE inhibitors is believed to be a promising strategy for preventing diabetic complications.

To probe the potential role for Totala mineraloid isolate in alleviating NEG, two distinct methods were used. The first method, based on the quantification of non-tryptophan fluorescence, detects chemical structure modification, which in this model consists of a conformational shift in albumin due to its glycation [20]. We found that, similarly to aminoguanidine, Totala partially suppressed the increase of non-tryptophan fluorescence of albumin incubated with glucose, which indicates a lower rate of albumin glycation (Figure 2). This was further confirmed by the dot blot method, in which AGEs were quantified with specific monoclonal antibodies (Table III). Together, these data suggest a potential role of Totala in controlling NEG and downstream complications *in vivo*, through mechanisms yet to be determined, and possibly involving mineral supplementation and chelation [21].

Given the promising anti-diabetic effects of this mineraloid isolate identified *in vitro*, we further investigated its activity in genetically diabetic mice.

It was found (Table V) that Totala administered in drinking water slowed weight gain in diabetic mice in a dose-dependent fashion. This effect was not due to diminished fluid intake and thus could result either from reduced food intake or from increased metabolism. The increased metabolism hypothesis is in agreement with *in vitro* results reported here.

In parallel with the weight gain, all groups registered an increase of blood glucose level, however this increase was reduced by about 60 % in both groups drinking Totala-supplemented water. This result is strongly indicative of the anti-diabetic potential this mineraloid isolate could have in humans as, for example, a nutrition supplement. This is especially important in the context of type II diabetes being a disease strongly influenced by the diet. As documented by a recent U.S. National Institutes of Health (NIH) clinical study, nutrition supplementation, delivering mild but sustained amelioration of blood sugar levels may result in a better overall morbidity and mortality control than the aggressive pharmacological treatment [22].

Besides the positive impact on blood sugar, Totala-drinking groups showed a 3.5 % decrease in the amount of glycated hemoglobin (HbA1C), which reflects long-term glucose trends and is not subject to daily blood glucose fluctuations [23–24]. This slight decrease was not statistically significant ($p > 0.05$), however, the 31-day timepoint for this measurement was not optimal for HbA1C quantification and thus no definitive conclusions can be made. Longer treatment could possibly result in bigger differences, as the optimal time period for HbA1C measurement in mice is 12–16 weeks [25]. Indeed, because the aver-

Table V: Effect of Totala isolate on body weight, blood glucose, alkaline phosphatase and albumin/globulin ratio in genetically diabetic mice

Test Material	Body Weight Increase (D31–D0) (g)	SEM	p value vs. water	Blood Glucose Increase (D31-D0) (mg/dl)	SEM	p value vs. water	AP Day 31 (U/L)	p value vs. water	A/G Ratio Day 31	p value vs. water	HbA1C Day 31 (%)	SEM	p value vs. water
water	12		N/A	151	44	N/A	171	N/A	1.8	N/A	6.9	0.1	N/A
Totala 0.5 mg/ml	11	1 1	0.25	58	34	0	143	0.03	2.1	0.049	6.6	0.3	0.17
Totala 3 mg/ml	9	1	0.02	61	35	0	148	0.03	2.4	0.008	6.6	0.2	0.3

age erythrocyte life span in mice is 40 days [26], the HbA1C measurement on day 31 reflects the mean daily blood glucose concentration between day 1 and day 15 of the treatment. Therefore, any improvements in the second half of the study are not detectable.

Of interest is the significant decrease of alkaline phosphatase (AP) and increase of albumin/globulin (A/G) ratios in Totala-drinking groups. The decrease of AP may be associated with an improvement in diabetic condition, as increased AP has been long known to be a marker of diabetes mellitus [27–28]. Similarly, an increased ratio of albumin to globulin (A/G) may indicate an attenuation of the prothrombotic environment, characteristic of the physiopathology of diabetes [29]. Taken together, these data demonstrate an overall anti-diabetic functionality of Totala administered in drinking water to diabetic animals.

To our best knowledge, this is the first description ever of a fossil material having anti-diabetic activity in pre-clinical models. The fact that this mineraloid isolate has been found to possess such complex, medically relevant effects underlines the usefulness of exploring unusual sources when mining for new actives. Leonardite is associated with deposits of a hundred million tons of lignite and sub-bituminous coals; e. g. in North Dakota, USA, Alberta, Canada, and Achlada and Zeli in Greece, as well as areas in Turkey and China. Totala is isolated from byproducts of leonardite extraction, which otherwise would have been discarded, and therefore its source is not only abundant, but its production allows fuller use of our natural resources.

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