

Benefits of Natural Compounds in Diabetes Complications by Prevention and Reversing Protein Glycation and Protecting Cells against Oxidative Stress

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Abstract Background: Diabetes is a global health crisis. Chronic hyperglycemia leads to the formation of glycosylated proteins known as Advanced Glycosylated End Products (AGEs) which damage multiple organs resulting in diabetes-related retinopathy, atherosclerosis, neuropathy and other pathologies. Therefore, prevention and reversal of protein glycation is an important aspect in diabetes management, especially involving natural, non-toxic approaches. Methods and Results: We tested the efficacy of natural compounds, including Krebs cycle mediators and amino acids in different aspects of diabetes. Firstly, we demonstrated that these compounds work individually and synergistically to prevent glycation of bovine serum albumin caused by glucose and fructose. Secondly, our study showed that the test compounds reverse glycation of L-lysine by converting Nε-(1-Carboxymethyl)-L-lysine to L-lysine in vitro, implying that natural removal of AGEs is possible. We also demonstrated that combinations of natural compounds can address important aspects of diabetes metabolism, such as impaired mitochondrial bioenergetics and cellular damage by oxidative stress. Conclusion: Our data show that the combination of natural compounds stimulates mitochondrial activity and mitobiogenesis in skeletal muscle cells, which are impaired in diabetes and linked to the development of diabetic cardiomyopathy. In addition, these natural compounds protect microglial cells from oxidative stress –a common hallmark of hyperglycemia implicated in neuropathy, vascular systems damage and other pathologies. Our data demonstrate that a combination of selected natural compounds has superior biological efficacy compared to its individual components in important metabolic aspects of diabetes and should be further evaluated as a non-toxic alternative in diabetes treatment and/or management.

Keywords: diabetes, Advanced Glycation End products (AGEs), micronutrients

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1. Introduction

Advanced Glycation End products (AGEs) are modifications of proteins or lipids which form *in-vivo* in hyperglycemic environments and during aging. They contribute to the pathophysiology of diabetes, cardiovascular disease, Alzheimer's, and accelerate aging. [1,2] Many of the negative effects of AGEs occur via AGE activation of a cell receptor called Receptor of Advanced Glycation End products (RAGE). [3] Serum levels of soluble RAGE have been implicated in many aspects including ovarian dysfunction leading to infertility. [4]

AGEs modified proteins or lipids are a result of nonenzymatic glycation and oxidation after their contact with sugars, notably fructose and glucose. [5] AGE formation, or the Maillard reaction, begins from Schiff bases and the Amadori product, produced by reaction of the carbonyl group of a reducing sugar, like glucose, with

proteins, lipids, and nucleic acid amino groups. [6,7] These highly reactive intermediate carbonyl groups, known as alpha-dicarbonyls or oxoaldehydes, can react with amino, sulfhydryl, and guanidine functional groups in proteins resulting in cross-linking and denaturation of proteins. [8] In addition, the alpha-dicarbonyls can react with L-lysine and L-arginine functional groups on proteins, leading to the formation of stable AGE compounds, such as Nε-(1-Carboxymethyl)-L-lysine (CML), which are non-fluorescent AGEs. [9] CML accumulation is also known to occur in tissues when AGE-containing foods are consumed. [10] CMLs form *in-vitro* from low density lipoproteins (LDL) incubated with copper ions and glucose and therefore are believed to be both lipid and protein adducts. [11] Their presence has been linked to the progression of various diabetic complications and some neurodegenerative diseases. [4,12] CML is one of the most abundant AGEs found in the renal compartment and is linked to the loss of kidney function in chronic kidney disease. [13]

Once AGEs are formed, they are considered essentially irreversible. [14] In this study, we address the basic biological process in AGE formation and reversal that involves CML, which is a lysine-derived AGE. Therefore, we tested several natural compounds individually and as mixtures to address their efficacy in both prevention and reversal of AGE formation *in-vitro*. The AGE reversal assay was based on a method to convert CML which is an AGE product and a known physiological ligand of RAGE to L-lysine. [15] Thus, the conversion of CML to L-lysine would represent the reversal of the glycation modification. The assay was adapted from a publication where they used a modified *E. coli* enzyme to cleave CML. [16] We applied natural compounds, amino acids, and Krebs cycle components, to obtain the same reversal of glycation.

Further, using an established protocol for AGE formation we evaluated the efficacy of individual ingredients and their combinations in preventing glycation of Bovine Serum Albumin (BSA) by both glucose and fructose. We used the xanthine oxidase-based assay described by Marques et al. to chemically glycate BSA and inhibit this process using natural ingredients. [17]

Mitochondrial metabolism is another important aspect in diabetes as it is a major source of bioenergy in the form of adenosine triphosphate (ATP) and is considered as master regulator of insulin secretion. Its dysfunction has been implicated in the pathogenesis of insulin resistance, the hallmark of type 2 diabetes. Both mitochondrial activity and their biogenesis are essential in overall metabolism. Since mitochondrial pool is inherited maternally, it can only increase by growth and division of preexisting mitochondria. Our evaluation of mitobiogenesis was conducted using heart muscle cells and based on detecting Cyclooxygenase-1 (COX-1) activity (in respiratory chain Complex IV) and Succinate Dehydrogenase (SDH) subunit A, which is another mitochondrial membrane protein as well as a tumor suppressor. [18]

Oxidative stress plays a pivotal role in the development of diabetes complications including diabetes-specific pathology in the retina, renal glomerulus, and peripheral nerve and accelerated atherosclerosis which affects arteries that supply blood to the heart, brain, and lower extremities. Oxidative stress is also implicated in development of cancer, neurodegenerative diseases (Alzheimer's and Parkinson's), Amyotrophic Lateral Sclerosis, pulmonary diseases, and various allergies, among others. Therefore, there is an urgent need to develop effective, economic, and safe approaches to protect cells against oxidative stress.

This study shows how individual micronutrients, and their specific combinations affect important aspects associated with hyperglycemia and diabetes. [19,20] The results demonstrate that a combination of natural compounds is more efficient than its individual ingredients in addressing important metabolic aspects relevant in diabetes, such as preventing and potentially reversing AGE formation, promoting mitobiogenesis in the muscle cells and protecting microglial cells from death caused by oxidative stress.

2. Materials and Methods

All test ingredients were solubilized in dimethylsulfoxide

(DMSO) (MilliporeSigma, Massachusetts, USA). The Mix was formed by combining all ingredients in Table 1 in equal amounts. A subset for the Mix ingredients designated as "Core" is formed by six test ingredients: sodium pyruvate, alpha-ketoglutaric acid, niacinamide, L-proline, L-glutamine, and L-citrulline. The nutrient composition of the Mix and Core is presented in the respectively labelled columns of Table 1.

Table 1. Test ingredients combined in the Core (left column) and Mix (right column)

Core	Mix
Alpha-Ketoglutaric Acid	Alpha-Ketoglutaric Acid
Niacinamide	Niacinamide
L-proline	L-proline
L-glutamine	L-glutamine
L-citrulline	L-citrulline
Sodium Pyruvate	Sodium Pyruvate
	Pantothenic Acid
	L-cysteine
	Magnesium Malate
	Magnesium Citrate
	L-methionine

Other reagents used are Nε-(1-Carboxymethyl)-L-lysine (CML) (Cayman Chemical Company, Michigan, USA), 2,4-dinitrophenylhydrazine (2,4-DNPH) (MilliporeSigma, Massachusetts, USA), hydrochloric acid (Thermo Fisher Scientific, Massachusetts, USA), Phosphate Buffered Saline (PBS), hypoxanthine, and xanthine oxidase (MilliporeSigma, Massachusetts, USA), KH₂PO₄, K₂HPO₄, ethylenediaminetetraacetic acid (EDTA), ferric chloride, bovine serum albumin (BSA), glucose, fructose, and sodium hydroxide (NaOH) (Thermo Fisher Scientific, Massachusetts, USA).

Cell Lines

Rat cardiomyoblasts (H9c2) derived from embryonic BD1X rat heart tissue that exhibit many of the properties of skeletal muscle, were purchased from ATCC (Virginia, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Massachusetts, USA), and 1% penicillin-streptomycin (PS) (MilliporeSigma, Massachusetts, USA).

Microglial Cells (IMG) were purchased from Kerast (Massachusetts, USA). It is an immortalized microglial cell line isolated from the brains of adult mice. Cells were maintained in DMEM supplemented with 10% FBS and 1% PS.

Glycation Reversal Assay

For the AGE product CML cleavage reaction, 90 µl of 100 mM CML in PBS was incubated with 10 µl test ingredients diluted in PBS in Eppendorf tubes (1.5 mL). Incubation was carried out at 37°C and 300 rpm shaking for 4 hours. Next, 50 µL of 2,4-DNPH solution (1 mM in 1 N HCl) was added to the mixture, with 350 µL of 0.6 N NaOH. This was followed by incubation at 37°C for 10 minutes. After this, optical density was measured at 445 nm for a 150 µl volume from the reaction mix.

The readout of this CML-dissolution assay is based on the cleavage of CML which results in the formation of L-

lysine, hydrogen peroxide and pyruvic acid. Using the α -ketoglutaric acid derivatization assay described in (8), sodium pyruvate reacts with 2,4-DNPH to form an adduct - the 2,4 DNPH-pyruvic acid adduct. This adduct can be modified by a base (i.e., NaOH), and detected by measuring optical density (OD).

Glycation Prevention Assay

Glycation prevention reaction buffer contained the following ingredients: 500 mL of phosphate buffer ($\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$, 0.1 M), EDTA (73 mg), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (33 mg), and hypoxanthine (20.5 mg).

The following reaction mixtures were added to Eppendorf microfuge tubes (1.5 mL): 94 μL BSA (16 mg/mL in buffer), 25 μL glucose (1.67 M in buffer), 10 μL xanthine oxidase (XO, 18 mU in buffer), 10 μL test ingredients (40 mg/mL stock in DMSO), 882 μL glycation prevention reaction buffer.

The negative control was set up lacking BSA and test ingredients, and only containing glucose or fructose and XO. Positive control lacked Test ingredients and contained BSA, glucose or fructose, XO, and test ingredients. Every reaction was set up in duplicate. Tubes were incubated in the dark at 37°C for 5 days with shaking at 450 rpm. After incubation, aliquots were transferred to black 96-well plates. Each reaction tube was aliquoted into 3 wells at 290 μL each, to be analyzed in triplicate. Total fluorescence intensity was measured with excitation and maximum emission at 360 nm and 460 nm, respectively.

Cell Protection from Oxidative Stress

IMG cells were grown to confluency and pretreated with test compounds dissolved in DMEM supplemented with 10% FBS for 24 hours at 37°C. Media was removed and cells were exposed to hydrogen peroxide for 1 hour at 37°C. After one hour, H_2O_2 was removed, and cells were incubated in DMEM supplemented with 1% BSA for a further 24 hours at 37°C, to allow them to stabilize. Subsequently, cell viability was assessed using Alamar Blue Cell Viability Reagent (Thermo Fisher Scientific, Massachusetts, USA). Alamar Blue Reagent is an oxidized form of redox indicator that is blue in color. When incubated with viable cells, the reagent changes color from blue to red and can be measured by absorbance at 570 nm.

Mitobiogenesis

MitoBiogenesis™ In-Cell ELISA Kit (Colorimetric) was purchased from Abcam (Cambridge, UK). H9c2 Cells were grown to confluency in 96-well plates and treated with test ingredients for 24 hours at 37°C. Medium was removed and cells were washed with PBS provided in the kit. The cells were then fixed and processed as per the protocol provided with the kit. This assay was used to quantify two mitochondrial proteins: subunit I of Complex IV (COX-1) and the 70 kDa subunit of Complex II (SDH-A). No treatment control was used to show relative protein levels in each case.

3. Results

Natural Compounds Prevent Protein Glycation

Figure 1A and Figure 1B show that incubation of BSA in the presence of glucose and fructose, respectively,

increases the formation of AGE-BSA. In the presence of fructose (Figure 1B) there was a higher level of protein glycation observed compared to glucose (Figure 1A). Individual ingredients tested in this experiment showed different efficacy in preventing AGE-BSA formation in the presence of both sugars. The highest efficacy in preventing BSA glycation was observed when these ingredients were applied in combinations indicated as the Mix (11 compounds) and Core (six compounds). As such, in glucose-driven glycation the Core combination of six ingredients was effective in preventing AGE-BSA by 54% and the Mix by 64% (Figure 1A). Fructose promoted glycation of BSA was inhibited by the Core combination by 57% and by Mix by 73% (Figure 1B). Fructose+XO also shows significant fluorescence level.

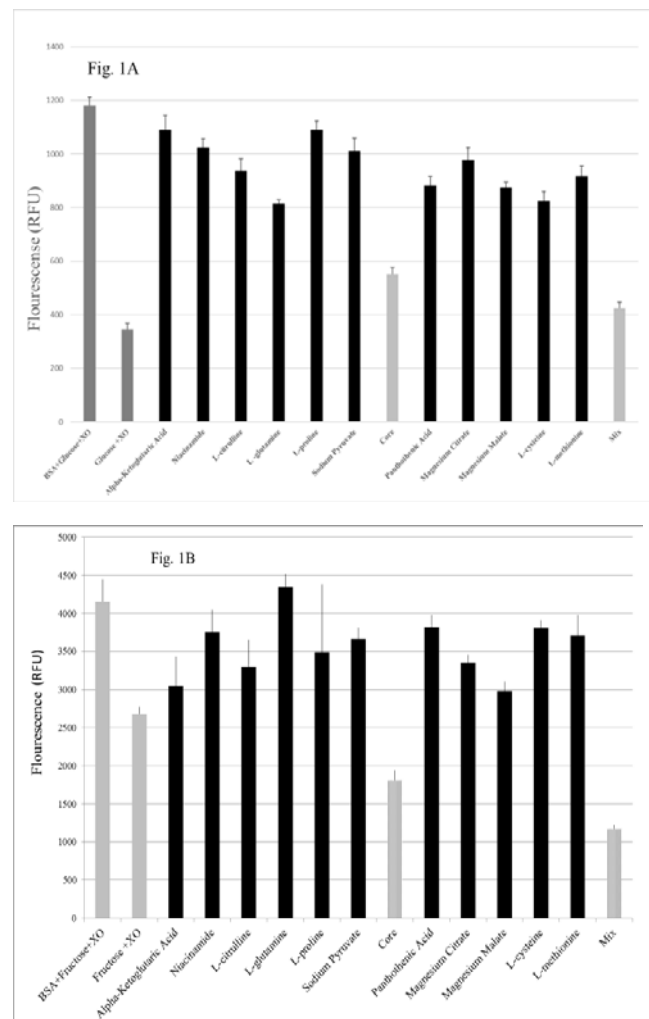


Figure 1. (A, B): Effects of individual natural compounds and their combinations as Mix and Core on AGE-BSA formation in the presence of glucose (Figure 1A) and fructose (Figure 1B). Positive control for AGE-BSA formation is the combination of glucose (Figure 1A) or fructose (Figure 1B) with BSA and Xanthine Oxidase (XO). Core consists of glucose or fructose, BSA and XO together with sodium pyruvate, alpha ketoglutaric acid, niacinamide, L-proline, L-glutamine and L-citulline in equal amounts and Mix contains glucose or fructose, BSA and XO, all Core ingredients together with pantothenic acid, magnesium citrate, magnesium malate, L-cysteine and L-methionine. Negative controls are shown using glucose+ XO (Figure 1A) or fructose+XO (Figure 1B). Error bars represent standard deviation

Natural Compounds in Promoting Reversal of Glycation Process

The potential of natural compounds potential for their *in vitro* efficacy in reversal of AGE was tested based on a method to convert N ϵ -(1-Carboxymethyl)-L-lysine (CML), which is an AGE product and a known physiological ligand of RAGE to L-lysine. Thus, the conversion of CML to L-lysine would represent the reversal of the glycation modification. The experimental procedure is described in detail in Materials and Methods.

The results in Figure 2 show that L-citrulline and sulfur containing amino acids as well as L-methionine and L-cysteine tested individually at two concentrations, were not effective in releasing free L-lysine from CML. However, these amino acids combined with other components in the Mix and Core were very effective in supporting the glycation reversal process. As such, the combination of these amino acids with L-proline, L-glutamine, sodium pyruvate, alpha ketoglutarate, magnesium, pantothenic acid, and niacinamide applied at 22 μ g/ml could release about 4 times more free L-lysine compared to control, and at its 440 μ g/ml concentration the conversion of CML to L-lysine was about 10 times more effective than a control. A smaller subset of test compounds (Core) containing sodium pyruvate, alpha ketoglutaric acid, niacinamide, L-proline, L-glutamine, and L-citrulline applied at 12 μ g/ml, was also effective in increasing CML reversal by about 500% compared to control.

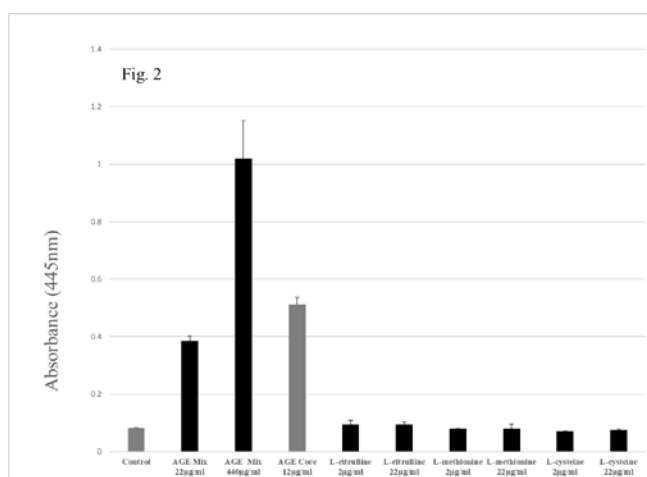


Figure 2. Release of native L-lysine from CML in the presence of selected individual compounds and nutrient combinations in Mix and Core evaluated by change in the absorbance at 445nm. AGE Mix was applied at 22 μ g/ml and 440 μ g/ml. Core was applied at 12 μ g/ml. Each amino acid (L-methionine, L-cysteine, and L-citrulline) was applied at 2 μ g/ml. Error bars represent standard deviation

Natural Compounds Promote Mitobiogenesis:

Mitochondrial biogenesis was evaluated by changes in two mitochondrial enzymes: succinate dehydrogenase (SDH), which forms a part of mitochondrial Complex II A and COX-1 present in mitochondrial Complex IV. As such, we evaluated the effects of selected natural compounds individually and their combinations in Mix and Core on the levels of SDH and COX-1 in the heart muscle cells. The results are presented in Figure 3A and Figure 3B.

The results on Figure 3A show that among individual compounds, sodium pyruvate, pantothenic acid, magnesium citrate, and magnesium malate had the most pronounced stimulatory effects on SDH in the cardiac muscle cells by increasing its levels from 54% (sodium

pyruvate) to 86% (magnesium malate), compared to control. The highest stimulatory effect on SDH was observed when all test ingredients were combined as a Mix, with 118% increase. Core ingredients stimulated SDH by 68% compared to control. COX-1 levels in the heart muscle cells increased in the presence of niacinamide, magnesium malate, and L-methionine by 52-58% and L-cysteine by 41%, compared to control (Figure 3B). The highest stimulatory effect on COX-1 levels was achieved in the presence of Mix (79%) with Core having a more moderate effect of 42% increase.

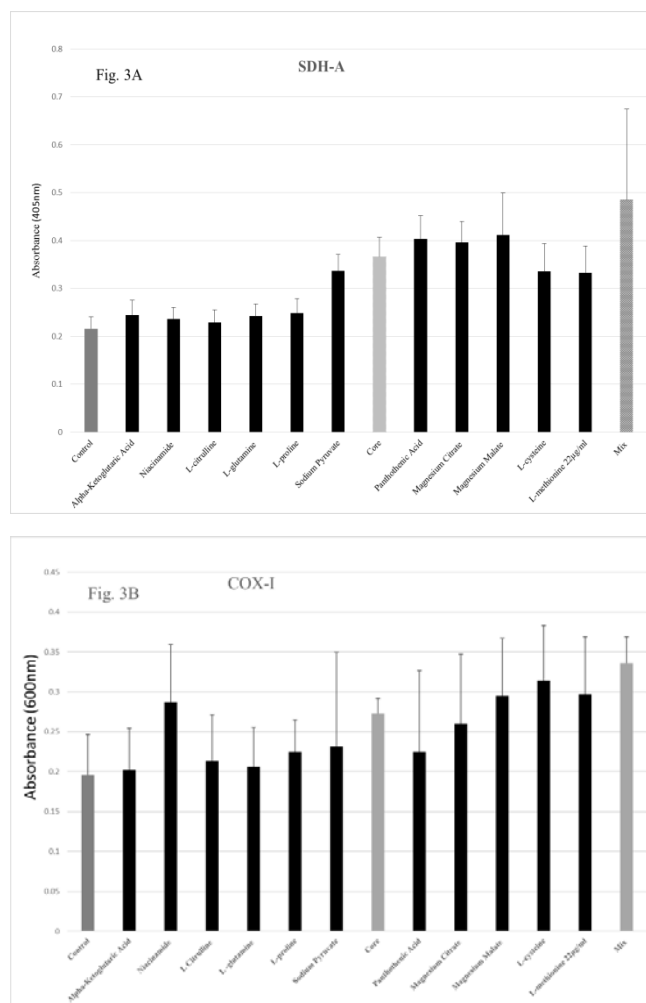


Figure 3. (A, B): Effects of individual ingredients, and their combinations as Mix and Core on SDH. (Figure 3A) and COX-1 levels (Figure 3B) in the heart muscle cells. H9c2 cells were treated with various ingredients at 1 μ g/ml per ingredient. Core was applied at 6 μ g/ml and Mix at 12 μ g/ml. After 24 hours of treatment, cells were fixed in formaldehyde and processed using MitoBiogenesis™ In-Cell ELISA Kit (Colorimetric) purchased from Abcam (Cambridge, UK). Error bars represent standard deviation.

Natural Compounds Protect Glial Cells from Oxidative Damage

We tested protective effects of individual natural compounds and their combinations on the survival of microglial cells exposed to oxidative stress generated by hydrogen peroxide. The results on Figure 4 show that in the presence of individual ingredients the survival of cells exposed to hydrogen peroxide increased. The lower antioxidant protection compared to other ingredients was observed in the presence of L-citrulline and L-glutamine.

About 50% of cells exposed to magnesium citrate, magnesium malate, and L-methionine survived in the presence of 1.5 mM H_2O_2 . The highest cell protection against hydrogen peroxide was achieved when the nutrients were combined either as Core or a Mix with 67% and 71% surviving cells, respectively.

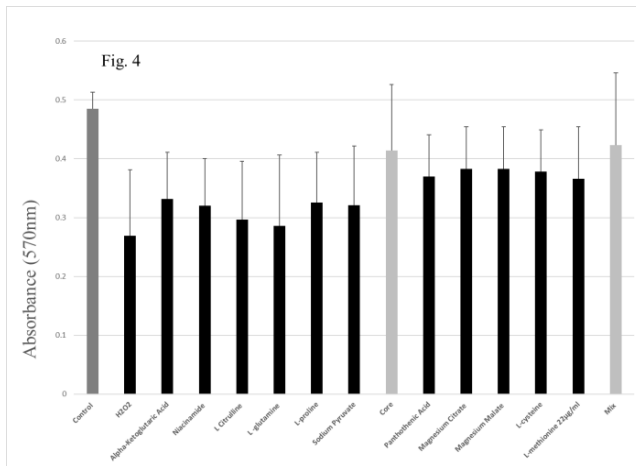


Figure 4. Effects of individual ingredients, and their combinations as Mix and Core on survival of microglial cells exposed to 1.5 mM H_2O_2 for 1 hour. Viable cells were stained with Alamar Blue. Cell viability was expressed as optical density at 570 nm. Control represents optical density of stained viable cells with no treatment. Error bars represent standard deviation

4. Discussion

Chronic diabetes involves multiple metabolic processes affecting various organs in the body. In this study, we sought a natural approach towards diabetes management by preventing and reversing some of the complications associated with diabetes. Therefore, we evaluated protein glycation (AGE), mitochondrial biogenesis (cellular bioenergy), and protection from oxidative nerve damage as key parameters associated with diabetic complications. [19,20] The test ingredients included the mediators of the Krebs Cycle (alpha-ketoglutaric acid, malate, pyruvate, citrate, magnesium) involved in mitochondrial bioenergy, sulfur containing amino acids (L-methionine, L-cysteine, L-proline), as well as L-glutamine and L-citrulline (the precursor for L-arginine) known for improving athletic performance and wound healing, a common complication of diabetes. [21,22]

In the AGE reversal process, we first evaluated the effects of L-citrulline, L-methionine and L-cysteine applied at 2 µg/ml and 22 µg/ml. Chilujkuri et al. reported that sulfur-containing amino acid, taurine, could inhibit Schiff base formation between proteins and reactive sugars and cysteine could inhibit protein glycation by trapping dicarbonyl compounds and activating the glyoxalase system. [23] Also, sulfur containing dipeptides derived from fresh garlic, such as γ -glutamyl-methylcysteine (γ -GMC) and γ -glutamyl-propylcysteine (γ -GPC), exhibited antiglycation activity, which was attributed to their scavenging abilities. [23] Our results show that as individual compounds L-citrulline, L-methionine or L-cysteine were not effective in reversing glycation by releasing free lysine from CML. Conversely, the combination of these amino acids with other

compounds in the Mix and Core showed significant effects in catalyzing CML conversion to free L-lysine with 440 µg/ml concentration being more than twice effective than 22 µg/ml. This indicates an important aspect in understanding the AGE reversal process as it demonstrates that combinations of natural compounds can be effective in reversing glycation which is considered mostly irreversible.

In addition to AGE reversal potential, the Mix, Core and some of their individual components were also effective in preventing the glycation of BSA at varying levels with the Mix and Core being the most effective. Our results show that our negative control Fructose+XO (lacking BSA) has nearly two times higher fluorescence emission than Mix or Core treated samples which contain BSA. We hypothesize that fructose could be glycosylating XO in the absence of test ingredients or exhibiting fluorescent emission of its own. The Mix and Core can work to prevent fructose glycation of both BSA and XO but that cannot be determined by this chemical assay.

These results further document that nutrient interactions play a critical role in final efficacy of various metabolic processes. Synergistically, the natural compounds we selected may help ameliorate negative effects of protein glycation, which is a hallmark of elevated serum sugar levels and responsible for many aspects of diabetic pathophysiology.

Currently, the anti-diabetic drug Metformin is known to mitigate the negative effects of AGEs via RAGE suppression and anti-inflammatory pathways. [24] Certain micronutrients like pyridoxamine and flavonoids e.g., rutin were also shown to have anti-AGE efficacy and antioxidant effects. [25,26] Here we demonstrate the beneficial effects of multiple nutrients that synergistically target both AGE formation as well as promote reversal, as one of the metabolic processes related to diabetic pathology.

Additionally, we also show that the Mix and all its components could protect microglial cells from oxidative stress. This has important practical applications since reduced nerve fiber density and neuronal damage are the markers of diabetic complication and neuropathy. [27] Both the Mix and Core compared to individual compounds demonstrate significant cell protective effects against oxidative stress.

The Mix and its ingredients also promoted mitobiogenesis. Diabetic myopathy is a risk factor in cardiac disease and affects 12% of all diabetic patients. [28] Our results show that both individual ingredients and their combination in Mix may help in diabetic cardiomyopathy, and support bioenergy production. Mitobiogenesis may also help in serum glucose utilization thus managing blood glucose levels. Increased bioenergy can help diabetic patients to exercise more effectively, thereby improving their insulin sensitivity. We have not evaluated mechanisms of action for our ingredients but, given our results, we may hypothesize that they exert their effects via the Krebs cycle and antioxidant properties. These and our previous results emphasize that a proper combination of ingredients can significantly enhance the desired metabolic effect with minimal doses of individual ingredients, allowing us to stay within safe consumption limits.

Given the serious impact of diabetes on public health and the fact that millions of prediabetes cases remain

undiagnosed it is important to develop natural, non-toxic and economic approaches that can be applied by large populations as prevention and adjunct treatment. Effective management of diabetes should involve comprehensive and pleiotropic control of various metabolic aspects of this pathology. The efficacy of a natural approach presented in this paper calls for pursuing further research and clinical application of these findings.

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Author Statement: Artificial Intelligence (AI) was not used in the preparation of this manuscript. This is an *in vitro* study. All test compounds, reagents, including biological reagents are commercially available and were purchased from suppliers mentioned in the Materials and Methods and Acknowledgement Sections in accordance with their academic and research use policies. We have no relationship with any of the suppliers.

Conflict of interest. This study was not requested by any non-profit or profit-oriented entity. No conflict of interest declared.

Abbreviations: Advanced Glycation End products (AGE); Receptor of Advanced Glycation End products (RAGE); Nε-(1-Carboxymethyl)-L-lysine (CML); low density lipoproteins (LDL); Bovine Serum Albumin (BSA); adenosine triphosphate (ATP); Cyclooxygenase-1 (COX-1); Succinate Dehydrogenase (SDH); Dimethylsulfoxide (DMSO); 2,4-dinitrophenylhydrazine (2,4-DNPH); ethylenediaminetetraacetic Acid (EDTA); Dulbecco's Modified Eagle's Medium (DMEM); Fetal Bovine Serum (FBS); xanthine oxidase (XO); γ-glutamyl-methylcysteine (γ-GMC); γ-glutamyl-propylcysteine (γ-GPC);

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