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Arginase Enzyme Activity in Human Serum as a Marker of Cognitive Function: The Role of Inositol in Combination with Arginine Silicate

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***Abstract*—**The purpose of this study was to evaluate arginase activity levels in response to combinations of an inositol-stabilized arginine silicate (ASI; Nitrosigine®), L-arginine, and Inositol. Arginine acts as a vasodilator that promotes increased blood flow resulting in enhanced delivery of oxygen and nutrients to the brain and other tissues. Arginase, found in human serum, catalyzes the conversion of arginine to ornithine and urea, completing the last step in the urea cycle. Decreasing arginase levels maintains arginine and results in increased nitric oxide production. This study aimed to determine the most effective combination of ASI, L-arginine and inositol for minimizing arginase levels and therefore maximize ASI's effect on cognition. Serum was taken from untreated healthy donors by separation from clotted factors. Arginase activity of serum in the presence or absence of test products was determined (QuantiChrom™, DARG-100, Bioassay Systems, Hayward CA). The remaining ultra-filtrated serum units were harvested and used as the source for the arginase enzyme. ASI alone or combined with varied levels of Inositol were tested as follows: ASI + inositol at 0.25 g, 0.5 g, 0.75 g, or 1.00 g. L-arginine was also tested as a positive control. All tests elicited changes in arginase activity demonstrating the efficacy of the method used. Adding L-arginine to serum from untreated subjects, with or without inositol only had a mild effect. Adding inositol at all levels reduced arginase activity. Adding 0.5 g to the standardized amount of ASI led to the lowest amount of arginase activity as compared to the 0.25 g, 0.75 g or 1.00g doses of inositol or to L-arginine alone. The outcome of this study demonstrates an interaction of the pairing of inositol with ASI on the activity of the enzyme arginase. We found that neither the maximum nor minimum amount of inositol tested in this study led to maximal arginase inhibition. Since the inhibition of arginase activity is desirable for product formulations looking to maintain arginine levels, the most effective amount of inositol was deemed preferred. Subsequent studies suggest this moderate level of inositol in combination with ASI leads to cognitive improvements including reaction time, executive function, and concentration.

***Keywords*—**Arginine, blood flow, colorimetry, urea cycle.

I. Introduction

ARGININE is an amino acid that helps the body build protein and acts as a vasodilator. It is a non-essential amino acid, meaning that the human body can synthesize arginine if it is not supplied by the diet or supplementation [1]. Arginine may assist in the prevention of heart and circulatory diseases, combat fatigue, and stimulate the immune system [2]. Arginine is a vasodilator that enhances the production of nitric oxide and relaxes blood vessels resulting in increased blood flow [3]. Arginine is also an important intermediate in the urea cycle and in the detoxification of nitrogenous wastes [4], [5]. In the urea cycle it is a precursor for the synthesis of other molecules including nitric oxide (NO), citrulline, agmatine, urea and ornithine [6]. Dubey et al. demonstrated that L-arginine administration in mice led to significant improvements in two cognitive tasks, the Morris Water Maze and the Passive Avoidance Test, as compared to placebo [7]. Clinical trials have confirmed the benefits of L-Arginine on cognition. Mone et al. tested the effects of L-Arginine in hypertensive elderly subjects and found significant improvements on the Montreal Cognitive Assessment (MoCA) test scores when comparing L-Arginine treatment versus placebo [8]. Other research suggests that L-arginine/NO pathway is altered in neurodegenerative and vascular dementia and affects biomarkers associated with the severity of cognitive impairment [9]. In addition, dysregulation of arginine metabolism is associated with increased cognitive impairment suggesting that improving arginine metabolic processes would be important for cognitive processing [10].

Inositol, a carbocyclic sugar alcohol (cyclohexanehexol) is closely related to glucose. There are nine inositol isomers, but myo-inositol is the most important for both plant and animal metabolism. In plants inositol is usually found as phytic acid. In animal tissues inositol is most commonly found in membranes and in the brain where it is most abundant [11]-[13]. Inositol is similar to other simple carbohydrates and the human body can synthesize it through consumption of other carbohydrates such as glucose [14]. It is naturally found in many food sources including whole grains, seeds, and fruits. According to the US FDA, inositol is included on the list of substances affirmed as generally recognized as safe (GRAS) [15].

Myo-inositol is a precursor in the phosphatidylinositol cycle (PI-cycle) which has a central role in cell signaling [16]. Inositol facilitates communication between brain neurons and many neurotransmitters require inositol to relay nerve signals [16]. It also assists in the functioning of serotonin and dopamine which are known to influence learning, memory, and pleasure [17], [18]. Inositol is also known to mediate cell signal transduction in response to a number of hormones,

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neurotransmitters, and growth factors. Recent work suggests that inositol is involved with excitatory synaptic functioning in Ip6k1-KO hippocampal neurons [19]. This may be of particular importance for the role of inositol on cognition since the hippocampus is well known to mediate learning and memory. It is also known that information from the sensory and association cortexes of the brain are routed through the hippocampus and then sent to the rest of the brain [20], [21]. Finally, the combination of substances, such as inositol with L-arginine, may lead to enhanced absorption and bioavailability of arginine [22].

It has been suggested that silicates can function to stabilize arginine residues on enzyme surfaces and that this would result in improved arginine functioning [23]. In addition, silicon both in drinking water and as a dietary component is reported to have beneficial effects on cognition and reduce the risk of Alzheimer's disease [24]-[26]. Inositol-stabilized ASI (ASI; Nitrosigine®) has previously been studied in healthy physically active adults for its NO production and cognitive enhancing properties [27]-[29]. However, the amount of inositol that would be most beneficial for the functioning of ASI has not been previously reported. Arginase is an enzyme that leads to the hydrolysis of arginine and produces urea and ornithine [30], [31]. Some have suggested that elevated arginase expression in vascular smooth muscle may be an important factor in development of vascular stiffness in response to injury or during aging [31]. It is possible that reducing arginase levels could be beneficial and that it could be a potential target in the treatment of cardiovascular disease [32]. The enzyme arginase is present in human serum, cells, and tissue. Arginase catalyzes the conversion of arginine to ornithine and urea, completing the last step in the urea cycle. The testing of arginase activity may utilize either human cells (such as red blood cells) or serum. We present here a study that aimed to determine the most effective combination of inositol with either ASI or L-arginine using arginase as a marker. We hypothesized that minimizing arginase levels would maximize the levels of ASI or L-arginine. We further hypothesized that the levels of arginase could underlie the reported benefits of ASI on cognition tests [22], [27], [33]. By decreasing arginase in the blood, arginine should be able to continue stimulating NO production and maintain blood flow more efficiently. By pairing together the appropriate dose of inositol with ASI, more inositol would be allowed to cross the blood brain barrier thereby improving additional cognitive outcomes.

II. Methods

Blood was drawn from a healthy donor who had not been exposed to supplemental L-arginine or supplemental ASI and then the blood was introduced into serum separator vacutainer tubes. Blood clotting took place for at least 30 minutes, after which the tubes were centrifuged for 15 minutes at 3,000 rpm to separate the clot from the serum. Ultrafiltration, using 10 kDa cut-off centrifugation filter units was performed since uric acid could be present at levels that could interfere with the detection of formation of uric acid by arginase during the assay. Thus, the uric acid needed to be removed from the sample before using the sample for testing of arginase activity. In a pilot assay, the detection of arginase activity in serial dilutions of serum versus red blood cell lysates was compared. This served to identify the sample baseline, and the optimal dilution of each sample, for further testing of the 10 ingredients (Table I). Each product was tested in three 2-fold dilutions. Separately, the optical density (OD; absorbance) of the test product dilutions were tested, such that the OD of products with an inherent color were subtracted from the uric acid readings of the same products/dilutions. Using the Millipore Amicon ultra-centrifugation filter units with a 10 kDa cut-off, uric acid was removed from the serum through a step-by-step process (Table II).

TABLE I
Ingredients and Blends Tested for Effects on Arginase Activity

Ingredients and blends for assay	Dose
ASI (Nitrosigine®)	1.0 g/L ASI (1500 mg dose)
L-arginine	0.4 g/L L-arginine (600 mg dose)
L-arginine + 0.25 Inositol	0.4 g/L L-arginine + 0.0625 g/L Inositol
L-arginine + 0.5 Inositol	0.4 g/L L-arginine + 0.125 g/L Inositol
L-arginine + 0.75 Inositol	0.4 g/L L-arginine + 0.1875 g/L Inositol
L-arginine + 1.0 Inositol	0.4 g/L L-arginine + 0.25 g/L Inositol
ASI + 0.25 Inositol (12:5 ratio of AS:I)	1.0 g/L ASI + 0.0625 g/L Inositol
ASI + 0.5 Inositol (2:1 ratio of AS:I)	1.0 g/L ASI + 0.125 g/L Inositol
ASI + 0.75 Inositol (6:5 ratio of AS:I)	1.0 g/L ASI + 0.1875 g/L Inositol
ASI + 1.0 Inositol (3:2 ratio of AS:I)	1.0 g/L ASI + 0.25 g/L Inositol

TABLE II
Steps for Removing Uric Acid from the Sample Serums

Step 1	100 µL serum was applied to the top chamber of each filter unit, and 400 µL distilled water (dH2O) was added.
Step 2	The filter units were centrifuged at 10,000 rpm for 10 minutes, resulting in approximately 400 µL fluid going through the filter, and leaving approximately 100 µL above the 10 kDa filter membrane.
Step 3	The clear fluid, containing low-molecular weight compounds, was discarded, and 400 µL fresh dH2O added to the top chamber.
Step 4	The centrifugation was repeated, and the clear fluid containing low-molecular weight compounds was discarded.
Step 5	The remaining ultra-filtrated serum in the top units were harvested and used as a source of arginase enzyme

In order to test the arginase enzyme activity levels in the absence versus presence of test products, a commercial kit was used (QuantiChrom™ arginase assay kit, DARG-100, Bioassay Systems, Hayward CA). This kit utilizes a chromogen that forms a colored complex specifically when urea is produced in an arginase reaction. The intensity of the color reflects the level of urea produced by arginase enzymatic activity and is directly proportional to the arginase enzyme activity in the sample. The kit is designed to detect and compare the arginase enzyme activity in serum samples from different donors, where the results depend upon a combination of arginase enzyme levels and arginase enzymatic activity. The standard serum from the donor was tested in duplicate and provided an excess of the enzyme substrate arginine and the co-factor magnesium. After incubation, a urea detection buffer was added, which under standard assay conditions terminated arginase activity and facilitated a color reaction to detect the level of urea in the samples. In parallel, additional duplicate sets of the same serum

samples were incubated for the same amount of time in the absence of external arginine and magnesium. After the urea detection reagent was added, the external arginine and magnesium was added, so the volume of each well was identical. We adapted the kit for testing of natural products as sources of arginine and/or magnesium. Because we use the same serum sample for the testing, the arginase enzyme level was the same for all samples, and the results reflect arginase enzyme activity rather than differing levels. We found that undiluted, ultra-filtrated serum was ideal for the assay, and that after the colorimetric development was completed, and before the samples are read by spectrophotometry, it was necessary to spin each sample at high speed to remove a fluffy precipitate and increase the precision of the optical reading (Fig. 1).

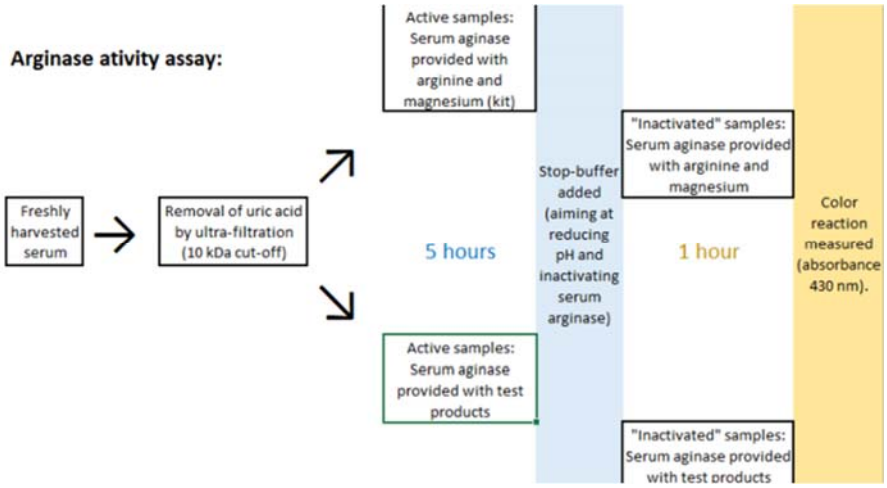

Fig. 1 Diagram showing the process for arginase activity determination

To verify that the freshly harvested serum used for this testing contained active arginase enzymes, serum arginase activity was tested using the standard kit components and the formula provided. Arginase activity (units per liter of sample (U/L)) was calculated with the formula indicated in Fig. 2. OD sample, OD blank, OD standard, and OD water are the optical densities (absorbances) of the active serum sample, the inactivated serum sample (blank), the urea standard (1 mM), and dH2O, respectively. The numbers 50 and 40 are the reaction and sample volumes, respectively, and *t* is the reaction time in minutes. Under these kit conditions with excess arginine and magnesium, the arginase activity of the serum sample used for this testing was 0.84 U/L. Ultra-filtrated serum was distributed in a flat-bottom 96-well microtiter plate with 40 µL serum per well. Four wells were plated per test product. Test products (10 µL/well) were added to the first two wells at the beginning of the assay, to provide sources of arginine to the samples. After the incubation was completed, the urea determination buffer provided with the arginase kit was added. After two minutes incubation to allow the buffer to inactivate arginase activity, test products were added to the two control wells for each quadruplicate set of samples. The urea determination reaction was allowed to take place for 1 hour, after which the OD (absorbance) was measured at 430 nm, using a plate-based spectrophotometer (PowerWaveX, BioTek Instruments).

$$\text{Arginase} = \frac{OD_{SAMPLE} - OD_{BLANK}}{OD_{STANDARD} - OD_{WATER}} \times [\text{Urea Standard}] \times 50 \times 10^3 / (40 \times t)$$

Fig. 2 Serum arginase activity formula

The addition of L-arginine to the serum triggered a very mild increase in urea levels. This was not significantly affected by addition of inositol at any of the 4 doses tested. The addition of ASI enhanced arginase activity. However, the urea determination buffer did not stop the enzymatic reaction, and color development was similar between the active test wells and the supposedly 'inactivated' test wells. The kit manual does not specify how the urea determination buffer inactivates arginase under the kit-based test conditions. However, based on the literature, we assumed that the mechanism involved lowering of pH to acidic conditions, which has been shown to reversibly inactivate arginase enzyme activity [4]. We tested the pH of the test product stock solutions and found that whereas all other test product stock solutions were neutral or mildly acidic (pH 5-7), ASI was strongly alkaline, with a pH well above 11. Therefore, we assumed that adding the urea detection buffer did not stop arginase activity in the control wells.

III. Results

When ASI was blended with inositol there were increases in urea levels above that for ASI alone. Note that the prominent increase for the lowest dose of inositol was associated with large standard deviations between the duplicate solutions. The apparent interference of ASI's pH with the inactivation of arginase in the control wells is taken into account in the data analysis, where the data are presented in two ways: Raw data (Absorbance 430 nm) are shown for all test products (Fig. 3) and Adjusted data: Data for non-ASI products and blends are shown where the matching product specific control wells are subtracted (Fig. 4). Data for ASI and blends that contain ASI are shown where the average of all non-ASI test product control wells was subtracted (Fig. 5). All tests showed some changes in

arginase activity demonstrating the efficacy of the method used. Adding L-arginine to serum, with or without inositol only had a mild effect.

Adding inositol to ASI at all levels reduced arginase activity. Adding 0.5 g to the standardized amount of ASI led to the lowest amount of arginase activity as compared to the 0.25 g, 0.75 g or 1.00 g doses of inositol (Fig. 5).

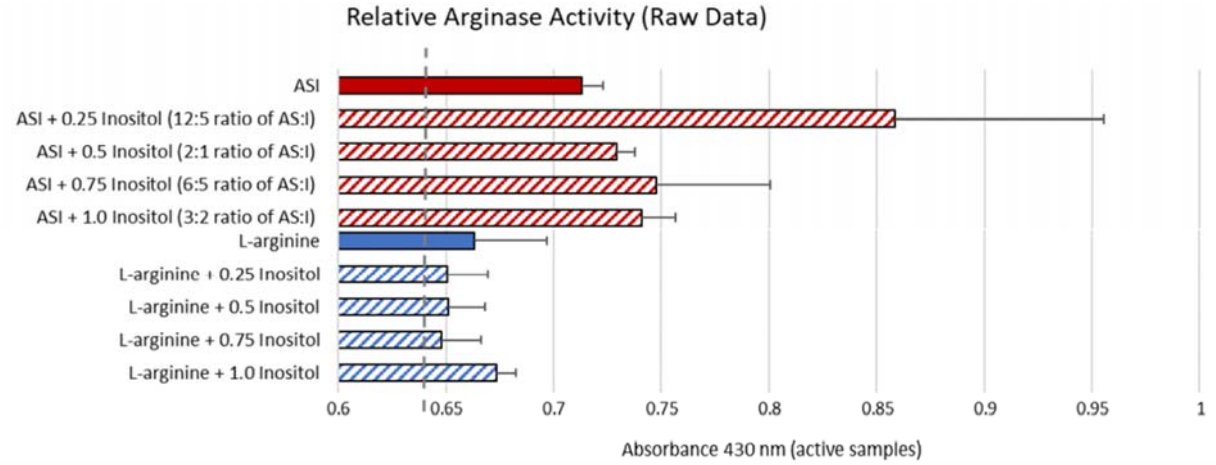

Fig. 3 The relative arginase activity is shown as the average + standard deviation of each duplicate test; The average absorbance of all control wells not containing ASI was 0.64 + 0.016, indicated by the vertical dashed grey line

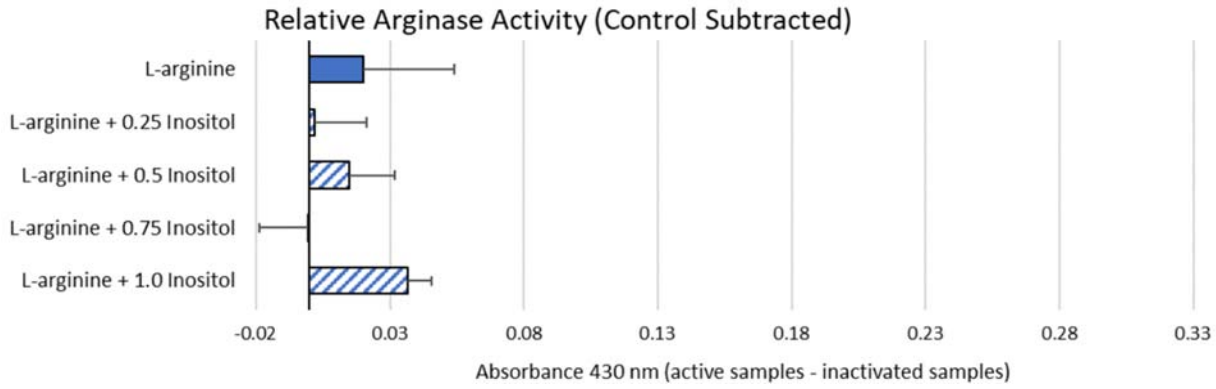

Fig. 4 The relative arginase activity is shown as the average ± standard deviation of each duplicate test, where the average of the matching product-specific duplicate control wells has been subtracted. To facilitate comparison, the scale on the X axis is the same as for the graph below that shows the ASI-containing products

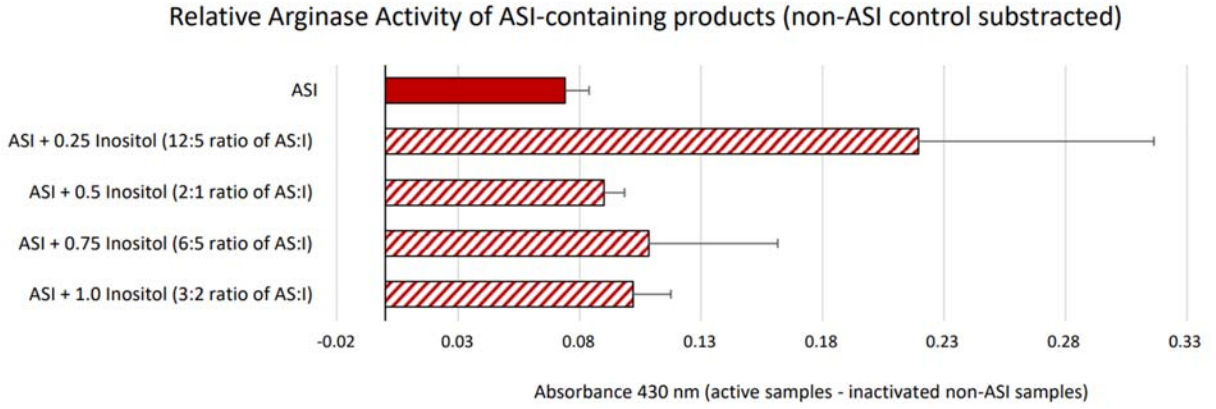

Fig. 5 The relative arginase activity is shown as the average + standard deviation of each duplicate test, where the average of all non-ASI control wells has been subtracted. To facilitate comparison, the scale on the X axis is the same as for the graph above that shows the non-ASI containing products

IV. Discussion

Our methods demonstrated the expected finding that adding inositol to ASI or L-arginine reduced the activity of arginase. However, the reduction of arginase was far more pronounced when inositol was combined with ASI compared to combining with L-Arginine. The most surprising finding was that adding 0.5 g of inositol to ASI led to the lowest amount of arginase activity compared to adding either more or less inositol. Presumably this lower level of arginase activity is most desirable and thus adding this moderate amount of inositol would maximize the blood flow benefits of arginine supplementation. We and others have subsequently tested this level of inositol in combination with ASI and demonstrated beneficial effects on blood flow and cognitive functioning [27], [34], [35].

While this study demonstrated that a moderate amount of inositol (0.5 g) was more effective at reducing arginase activity than either a higher or a lower dose this was only an *in vitro* assay and was not a clinical trial. To adequately assess if this unanticipated finding is applicable to improvement of human cognitive performance further research assessing different levels of inositol in combination with L-arginine or ASI would need to be carried out in human subjects. Since there have been clinical trials showing cognitive benefits of this level of ASI in combination with a human equivalent amount of inositol [27], [34], [35] this outcome is plausible but not yet proven.

Further research also needs to be carried out to determine which parts of the brain are most likely to be involved in the action of ASI with inositol. FMRI imaging especially of the hippocampus and frontal cortex might prove to be especially fruitful. The fact that nutritional interventions can be of benefit in cognitive performance is well documented [36], [37]. However, less explored is the interactions of specific nutrients and where in the brain they exert their actions. Further research in this area may allow more targeted interventions to improve cognitive outcomes.

Overall, the data from this study demonstrated the utility of a specific level of inositol leading to the enhancement of arginine levels and a reduction of arginase in an *in vitro* assay. Clinical trials have suggested that this specific inositol level in combination with ASI could lead to improved cognitive function but further research is needed.

V. Conclusions

All test products showed some level of increase in urea above the levels in control wells where arginase activity was inactivated. Adding L-arginine to serum, with or without inositol only had a mild effect. Adding ASI to the serum resulted in urea levels above that of pure L-arginine alone. A mild enhancement was seen when inositol was added. These data show that the pairing of inositol with ASI has direct effects on the extent of arginase enzyme activity. The level leading to the greatest suppression of arginase would logically be used in formulations. Formulations that incorporated this optimal level of inositol have been shown to led to significant improvements in cognitive tasks such as reaction time, executive function, and concentration [33]-[35].

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