An Amino Acid-Oligosaccharide Complex Increases Mineral Bioavailability

In Vitro

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Abstract

Limitations in the efficacy of mineral supplements include poor solubility at intestinal pH, low bioavailability and, particularly in the case with iron and copper, catalysis of oxidation. To address these limitations we tested a novel amino acid-oligosaccharide complex in a previously-validated in vitro digestion/absorption system. A proprietary multiminer-al/multivitamin admixture in either the novel organic or inorganic mineral forms were subjected to simulated gastric and intestinal digestion, followed by apical exposure to Caco-2/HT-29MTX enterocyte co-cultures. Bioavailability was assessed by minerals and ascorbic acid analysis of fluid from the basolateral culture compartment. Intracellular oxidative flux was measured using the fluorescent probe 5-(and-6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA). Compared to the inorganic mineral form, the organic matrix yielded significant increases in mineral solubility, and yielded several-fold increases in basolateral concentrations of all minerals tested (manganese 3.3x; copper 3.1x; iron 2.1x; and zinc 4.4x) compared to the inorganic matrix. Cells exposed to the organic formulation also had significantly lower levels of intracellular oxidation after a one-hour treatment. We conclude that the amino acid oligofructose formulation significantly increases mineral and ascorbic acid bioavailability and decreases intracellular oxidative stress in vitro.

Keywords: Minerals; Bioavailability; In vitro

Abbreviations: AAOS: Amino Acid Oligosaccharide; AAPH: 2,2'-Azobis-2-Methyl-Propanimidamide,Dihydrochloride; CM-H2DCFDA: 5-(and-6)-Chloromethyl-2, 7-Dichlorodihydrofluorescein Diacetate Acetyl; DMT-1: Divalent Metal Transporter 1; TEER: Transepithelial Electrical Resistance; REMS: Automated Tissue Resistance Measuring System

Introduction

Approximately 40% of the American population consumes supplemental vitamins on a daily basis [1], frequently formulations of multivitamins and minerals. Of the latter, iron is particularly important: iron deficiency anemia affects as many as two billion people world-wide [2]. If not treated, iron deficiency causes a variety of clinical morbidities, including delayed growth, delayed cognitive mental development, and comorbidities ranging from immune suppression to psychiatric disturbances [3-5]. Given the importance of dietary iron in human health, as well as other minerals and vitamins, food fortification and or vitamin supplements have been recommended as preferred approaches for preventing and eradicating iron and mineral deficiencies. While there are currently many different brands of commercially-available multivitamin/mineral formulations, most of the minerals are provided as inorganic complexes, including sulfates, chlorides, or oxides. Whether these formulations are truly bioavailable has not always been a marketing consideration. In fact, some vitamin and mineral complexes have a relatively low rate of uptake in vivo [6].

Generally minerals are consumed as part of a multivitamin/multi mineral formulation. Aside from poor solubility of some mineral forms at intestinal pH, they also may catalyze oxidation by generating free radicals and related oxidants, which can lead to degradation of accompanied vitamins and antioxidants and be harmful to general homeostasis. Indeed, fortification with mineral sources presents multiple challenges, not the least of which is the bioavailability [2-7]. From a pharmacological perspective, bioavailability is taken to mean the fraction of an oral dose that reaches systemic circulation [8]. This in turn is dependent on a variety of parameters, including solubility, enteral uptake, and first pass metabolism. Furthermore, mineral bioavailability may be reduced by mineral-specific factors such as complexation with other macro molecules, including proteins, oxidation (e.g., ferrous (Fe2+) to ferric (Fe3+) ion transformation), or interactions with mucin within the unstirred layer adjacent to absorptive cells. With respect to iron in particular, conversion from the ferrous to ferric state can generate hydroxyl free radicals and other oxidants leading to a wide range of oxidative events [9] thereby contributing to redox imbalances [10].

A number of studies have assessed iron solubility after gastrointestinal digestion, which turns out to be a reasonably reliable predictor of bioavailability, but this does not incorporate the possible effects of proteins [7]. Short of doing clinical human mineral uptake studies, a more realistic approach to the measurement of bioavailability would be to combine in vitro digestion with an appropriate model of the enterocyte absorptive lining of the gastrointestinal system. Robust models of this type have been developed, relying on the well-characterized Caco-2 enterocyte cell line [11,12]. More recently, Caco-2/HT-29MTX co-cultures have been developed that take advantage of the mucin-secretory activity of the latter cell line, with the aim of producing an endogenous mucin layer more representative of the apical microenvironment of the gastrointestinal epithelium [12-14]. We report herein on a new mineral delivery system: an amino acid-oligosaccharide (AAOS) complex that was developed with the aim of significantly increasing mineral solubility and, ultimately, bioavailability [15].

To facilitate side-by-side comparisons of this preparation with traditional inorganic preparations, we developed two formulations with identical vitamins and excipient matrices and amount of minerals, but only differed by mineral forms: Formulation 1 – AAOS, Formulation 2 – inorganic (sulfates, chlorides, and oxides).

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Goals

Our goals in this study were to: (i) compare the intestinal uptake of minerals from two identical multivitamin/mineral formulas that vary only in the form of the minerals; and (ii) to determine whether Formulation 1 (AAOS) abrogates oxidative stress. To these ends, we utilized a previously-validated in vitro digestion/intestinal cell culture system [12]. Our hypotheses were that: (1) AAOS increases both mineral solubility and intestinal uptake; and (2) AAOS mitigates oxidative stress in enterocytes.

Materials and Methods

Formulation

The amount of vitamins and minerals in both Formulations was based on FDA-recommended 100% daily intakes (Daily Values for Nutrition Labeling, 21 CFR §101.9(c) and CFR §101.36(b) (2) (ii) (B)) with the exception of calcium and magnesium. Micro-minerals such as copper, iron, zinc, manganese, chromium, and molybdenum were in the form of AAOS in Formulation 1 and in inorganic form in Formulation 2. Complete formulas are shown in Table 1. Vitamin A, vitamin C, vitamin D, vitamin E, vitamin K, thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, biotin, pantothenic acid, microcrystalline cellulose, croscarmellose sodium, silicone dioxide, magnesium stearate, and carnauba wax were purchased from Sigma Chemical Co, St. Louis, MO. Calcium carbonate, magnesium oxide, potassium iodide, copper sulfate, iron sulfate, zinc sulfate, manganese sulfate, chromium chloride, and sodium molybdate were purchased from Spectrum Chemical. AAOS minerals were prepared as previously described [15]. The USP grade amino acids were used for preparation of AAOS; glycine (CAS 56-40-6) and L-aspartic acid (CAS 56-84-8-L).

In vitro digestion protocol

In the gastric phase, the equivalent of 1 tablet of either Formulation 1 or Formulation 2 was dissolved in different concentrations in simulated gastric juice, which includes pepsin (Sigma Chemical, St. Louis, MO) (0.2 g pepsin dissolved in 5 mL of 0.1 M HCl, adjusted to pH 2.0. addition of HCl). Samples were incubated at 37°C in a shaking incubator for 30 min. Formation 1 samples were adjusted to pH 5.0 by addition of NaHCO3 and incubated for a further 30 min in the intestinal phase, 1.25 mL of pancreatin (Sigma)/bile (Sigma) were added to pH 7.0 by addition of NaHCO3 and to a final volume of 10 mL by 140 mM NaCl and 5 mM KCl solution. For bioavailability testing, tablets + digestive extracts were dissolved in 230 mL distilled, deionized water.

In vitro bioavailability testing

Co-culture cell model: To model the human intestinal mucosa, Caco-2BBE (ATCC, Rockville, MD) cells and HT29-MTX (clone E12) cells (a kind gift of Drs. Thomas Kissel (University of Marburg) and Per Artursson (University of Lund) were co-cultured at a ratio of 9:1, respectively, in either standard 96-well tissue culture-treated multi-well tissue culture plates (for toxicity testing) or in Transwell® plates (Corning, NY) pre-coated with 1% Matrigel® Matrix (BD Biosciences, Bedford, MA) (Figure 1) The latter is an artificial basement membrane substrate which contains matrix proteins (type IV collagen, laminin and fibronectin) that promotes cell adhesion. A total seeding density of approximately 5x10^4 cells mL^-1 was used in all culture assays; this density precluded changes in the original ratio of Caco-2BBE: HT-29MTX cells, since the latter have a shorter doubling time.

Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, St. Burlington, On) with 5 mL of a 100 U mL^-1 penicillin/ 100 µg mL^-1 streptomycin solution, 10 mL 2 mM L-glutamine solution, 5 mL 1 mM sodium pyruvate solution, 5 mL 10 µg mL^-1 human transferrin solution, and 60 mL 10% fetal bovine serum per 500 mL DMEM. Fresh culture medium was routinely changed every two days, and experiments initiated between 14 and 21 days of subculturing. Prior to experiments, culture confluency was confirmed optically and via measurement of transepithelial electrical resistance (TEER) using a REMS autosampler (WPI, Sarasota, FL). Transwell cultures were presumed confluent when a TEER value of 300 Ω cm^-2 was attained.

In vitro toxicity

To confirm that Formulation 1 is not cytotoxic, both digested and non-digested were incubated with co-cultures grown in 12-well

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount Per Serving</th>
<th>% Daily Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (as beta carotene)</td>
<td>5000 IU</td>
<td>100%</td>
</tr>
<tr>
<td>Vitamin C (as ascorbic acid)</td>
<td>60 mg</td>
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<tr>
<td>Vitamin D (as cholecalciferol)</td>
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<td>Vitamin E (as d-alpha tocopheryl succinate)</td>
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<td>100%</td>
</tr>
<tr>
<td>Vitamin K (as phytomenadione)</td>
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<td>100%</td>
</tr>
<tr>
<td>Thiamin (as thiamin HCl)</td>
<td>1.5 mg</td>
<td>100%</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.7 mg</td>
<td>100%</td>
</tr>
<tr>
<td>Niacin (as niacinamide)</td>
<td>20 mg</td>
<td>100%</td>
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<tr>
<td>Vitamin B6 (as pyridoxine HCl)</td>
<td>2 mg</td>
<td>100%</td>
</tr>
<tr>
<td>Folate (as folic acid)</td>
<td>400 µg</td>
<td>100%</td>
</tr>
<tr>
<td>Vitamin B12 (as cyanocobalamin)</td>
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<td>100%</td>
</tr>
<tr>
<td>Biotin (as d-biotin)</td>
<td>300 µg</td>
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</tr>
<tr>
<td>Pantothenic Acid (as calcium pantothenate)</td>
<td>10 mg</td>
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<tr>
<td>Calcium (as calcium carbonate)</td>
<td>100 mg</td>
<td>10%</td>
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<tr>
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<td>40 mg</td>
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<td>Iodine (as potassium iodide)</td>
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<tr>
<td>carnauba wax</td>
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</tr>
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</table>

**Table 1: Multivitamin/Multi Mineral Formulations.**
plates. Cells were gently trypsinized, and washed in fresh culture medium containing 5% fetal bovine serum. After aspiration, cells were centrifuged for 5 min at 100 g, the medium aspirated, and the cells resuspended in phosphate-buffered saline (PBS). Cells were then mixed with 1 part of 0.4% trypan blue to an equal part cell suspension and the mixture incubated 3 min at ~ room temperature. Approximately 50 µL of each trypan blue/cell mixture was added to a hemocytometer (American Optical, Company, Buffalo, NY), which was placed on a Zeiss IM35 inverted microscope with phase contrast optics. The unstained (viable) and stained (nonviable) cells were counted to obtain percentage of viable cells after treatment, the percentage of viable cells, which was calculated as follows: viable cells (%) = total number of viable cells per mL of aliquot/ total number of cells per mL of aliquot x100.

In vitro bioavailability

In vitro solubility was assessed as metals concentration in Formulation 1 and 2 after digestion. Samples were filtrated through 0.45µm filter, acidified with 0.2 M nitric acid and stored in glass vials until assay. Uptake and transport of metals were assessed with the Caco-2BBE/HT-29 co-cultures grown in Transwell plates. To initiate the experiments, cells were washed once in fresh MEM before adding 0.5 mL test solution containing either digested Formulation 1 or 2 to the apical culture surface. To initiate the experiments, cells were washed once in fresh MEM before adding 0.5 mL test solution containing either digested Formulation 1 or 2 to the apical culture surface. Cells were incubated with digested samples for 0, 1, 2, or 3 h at 37°C in a humidified atmosphere of 95% air and 5% CO2. Aliquots (1000 µL) of basal culture medium were removed and either digested for 1 h at 37°C in a humidified atmosphere of 95% air and 5% CO2. Each digested sample was treated with 1 mL of 0.2 M hydrochloric acid, vortexed then centrifuged for 1 min at 16000g in a micro-centrifuge tube. This solution was diluted to appropriate concentration and mixed with an equal amount of acid phosphotungstate. Samples were incubated for 30 min at room temperature, and optical density measured at 700 nm using a Beckman/Coulter DU520 spectrophotometer. Blanks consisted of 1% acetic acid. A standard calibration curve was obtained with serial dilutions of ascorbic acid (range 0.0-0.2 mg mL⁻¹).

Statistical Analysis

Results of oxidative stress assays are presented as mean ± standard error of the mean (SEM). To avoid Type I errors, a one-way analysis of variance was used to determine statistical significance (p<0.05).

Results

Viability testing

Cell viability data is presented in Figure 2. The positive control consisted of formalin-killed cells, while the negative control consisted of cells incubated in the basal culture medium. There were no significant differences in cell viability between Formulation 1 and Formulation 2 treated cells; nor were there significant differences between the treated- and the negative control (p<0.05).

Intracellular oxidation assay

Intracellular oxidation of the probe H₂DCF-DA was followed over one hour of exposure to either Formulation 1 or Formulation 2 at 37°C. As indicated in Figure 3, there was a low level of dye oxidation in test wells, which did not include any cells (Dye); in the absence of a cellular milieu, the non-de-esterified indicator dye (H₂DCF-DA) does not significantly oxidize in the presence of the free radical generator AAPH. Cell cultures treated with AAPH or AAPH + Formulation 2 yielded the highest levels of intracellular fluorescence. In contrast, intracellular oxidation was significantly lower in cell cultures treated with AAPH or AAPH + Formulation 1 after 30, 45 and 60 min incubation (p<0.05). The trend in greater fluorescence emission intensity in presence or absence of AAPH is not statistically significant. Cell cultures treated
incubation at both 1 h and 3 h. Formulation 1 yielded higher levels of Ascorbate after in vitro analysis of Ascorbate in Table 4. Due to the very low levels, most likely due to the degradation of the AAOS form significantly increases mineral bioavailability in our previous findings that AAOS blunts oxidative stress in vivo [15]. In this study, we used the oxidative stress probe CM-H$_2$DCFDA as an indicator of intracellular stress. While this thiol dye has been used in numerous studies to measure oxidative stress, we do acknowledge that the complexity of interactions between this dye and other thiols and thiol-derived radicals. Furthermore, pH variation and oxygen tension can confound interpretation of results of such assays [19]. Nevertheless, given the time course of our measurements, the results of both positive (AAPH-induced) and negative (no AAPH) controls, and the magnitude of differences between Formulation 1 and Formulation 2, we suggest that the effects of AAOF (Formulation 1) in blunting intracellular oxidative stress are robust.

Our intestinal cell culture system has been previously validated as an efficacious model of the in vivo state, and it is congruent with the finding that Formulation 1 increases mineral solubility. Similarly, our model provides support for the likelihood that the AAOF formulation enhances bioavailability. Further, increases in bioavailability would likely enhance systemic mineral uptake with the health benefits accrued as a result of meeting metabolic demands for these minerals. The increased mineral bioavailability conferred by Formulation 1 was at a lower level than would be predicted purely by the increased solubility in minerals in the Formulation 1 tablets. This is likely due to the fact that, while factors such as mucin enhance mineral transport, other morphological and molecular features of enterocytes regulate transmembrane flux, as depicted in Figure 4.

A key component of the current study is the side-by-side comparison of the two mineral formulations. The solubility and bioavailability of an individual mineral can be markedly different from its behavior as part of multivitamin/multimineral admixture [20]. Furthermore, it is difficult to compare different mineral forms using commercially available products; while mineral content may be the same, excipients (tablet fillers, tablets coating, flavoring and coloring additions) may have a dramatic effect on the properties exhibited by the minerals [21]. Formulations 1 (AAOS) and 2 (inorganic) differed only with respect to the mineral matrices; thus, in vivo solubility, bioavailability and biological effects most directly reflect this difference and no other confounding factors.

We propose that, by increasing the solubility of minerals, Formulation 1 also increases the amount of mineral-substrate available to transporters such as the apical DMT-1 and basolateral Ferroportin

with Formulation 1 alone had significantly lower fluorescence signals than cells treated with Formulation 2 at 30, 45 and 60 min incubation (p<0.05).

Solubility and bioavailability analyses

The relative solubilities of Formulation 1 vs. Formulation 2 are depicted in Table 2. Compared to the inorganic forms, Formulation 1 showed significant increases in mineral solubility. The absolute concentrations of minerals in the basolateral culture compartments are presented in Table 3. Again, there were significant, several-fold increases in the concentrations of all minerals added in the Formulation 1 supplement compared to the inorganic matrices.

Vitamin C analysis

Data from ascorbic acid (vitamin C) determinations are presented in Table 4. Due to the very low levels, most likely due to the degradation of Ascorbate in vitro, fluid samples collected from basolateral chambers were pooled. Formulation 1 yielded higher levels of Ascorbate after incubation at both 1 h and 3 h.

Discussion /Summary

AAOS form significantly increases mineral bioavailability in the Caco-2-HT-29MTX in vitro co-culture model. Furthermore, this form did not increase intracellular oxidative stress, which is consistent with...
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stress digestion/enterocyte co-culture system when compared in vitro in an

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References

Conclusion

AAOS significantly increases mineral and Ascorbate bioavailability in an in vitro digestion/enterocyte co-culture system when compared to an inorganic form. AAOS similarly decreases intracellular oxidative stress in vitro. Based on these findings and on our animal model, AAOS is a promising vehicle for mineral and multivitamin supplementation.

[22]. What is ultimately of interest is whether the increased solubility afforded by Formulation 1 (AAOS) enhances the bioavailability of physiologically-relevant minerals; based on our in vitro data, which simulates digestion and interaction with enterocytes coated with a mucin layer, we show herein that Formulation 1 does facilitate trans mural uptake of dietary metals.

While our evidence is encouraging, an important caveat is that in vitro models such as employed here are crucial adjuncts to clinical human testing, which ultimately remains the best method for determining the efficacy of mineral supplementation in vivo. Furthermore, a variety of factors can affect mineral uptake: age, gender, physiologic state, drug use [8]. Given these possible confounding variables, as well as the ethics and expense of human clinical trials, in vitro studies have an important role in helping us to understand factors that may impact the efficacy of vitamin and mineral supplementation in humans. Certainly, solubility is one such factor. By demonstrating that AAOS enhances not only solubility but also transepithelial uptake in a physiologically-representative model of the human intestinal mucosa, we suggest that our new matrix may overcome an important limitation in inorganic delivery systems.

Figure 4: Diagrammatic representation of transmembrane and intracellular ion transport mechanisms in enterocyte. DMT-1 = Divalent Metal Transporter-1. Bioavailability of iron depends on the solubility of iron, as well as the presence of appropriate transporters (DMT-1 and Ferroportin) as well as intracellular iron-binding proteins (ferritin).

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