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Effect of AstraGin® and AstraZyme® Lipid on the absorption of fatty acids and linoleic acid derived from NUTIVA organic hempseed oil(Hempco Canada) in human Caco-2 cells

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1. Abstract

The purpose of this study is to assess the effect of AstraGin® on the absorption of NUTIVA organic hempseed oil (Hempco Canada) derived fatty acids and linoleic acid in human small intestine Caco-2 cells. Pancreatin + pepsin+ bile extract or AstraZyme® Lipid+pepsin + bile extract were used to digest the ECO hemp oil and AstraGin® was added in differentiated Caco-2 cells for 24h prior to the studies.

AstraGin® increased total quantity of fatty acids absorption in Caco-2 cells by 28% and 38% after NUTIVA hempseed oil digested by pancreatin + pepsin+ bile extract and AstraZyme® Lipid+ pepsin + bile extract. AstraGin® increased initial transport rate of fatty acids absorption in Caco-2 cells by 100% and 120% after NUTIVA hempsed oil digested by pancreatin + pepsin+ bile extract and AstraZyme® Lipid+ pepsin + bile extract. AstraGin® increased total quantity of linoleic acid absorption in Caco-2 cells by 41% and 55% after NUTIVA hempseed oil was digested by pancreatin + pepsin+ bile extract and AstraZyme® Lipid+ pepsin + bile extract. AstraGin® increased initial transport rate of linoleic acid absorption in Caco-2 cells by 85% and 110% after NUTIVA hempseed oil was digested by pancreatin + pepsin+ bile extract and AstraZyme® Lipid+ pepsin + bile extract.

In summary, AstraGin® has shown to significantly increase fatty acids and linoleic acid absorption from NUTIVA hempseed oil that was digested by physiological digestive enzymes. Especially, AstraGin® displays its best effect on fatty acids and linoleic acid absorption of hemp oil that was digested by AstraZyme® Lipid.

2. Summary

Table1.Analysis of the total quantity of fatty acids and linoleic acid absorbed by Caco-2 cells in 20 minutes. Percent of fatty acids and linoleic acid absorption in NUTIVA hempseed oil digested with pancreatin + pepsin+ bile extract and AstraZyme® Lipid+ pepsin + bile extract. Treated hemp oil with pancreatin + pepsin+ bile extract was used as the control.

Group	Protease	Treatment	Fatty acids (%)	Linoleic acid (%)
Control	Pancreatin + pepsin+ bile extract	Maltodextrin	100.0±3.4	100.0±3.3
AstraGin®	Pancreatin + pepsin+ bile extract	**AstraGin®**	128.1±2.8**	141.3±6.7***
AstraZyme® Lipid + AstraGin®	**AstraZyme® Lipid**+ pepsin+ bile extract	**AstraGin®**	137.6±2.5**,##	155.4±3.8***,###

** p<0.01, when compared to control group

*** p<0.001, when compared to control group

##p<0.01, when compared to AstraGin® group

###p<0.001, when compared to AstraGin® group

Table2. Analysis of the initial transport rate of fatty acids and linoleic acid absorbed in Caco-2 cells in 5 minutes. Percent of fatty acids and linoleic acid absorption in NUTIVA hempseed oil digested with pancreatin + pepsin+ bile extract and AstraZyme® Lipid+ pepsin + bile extract. Treated hemp oil with pancreatin + pepsin+ bile extract was used as the control.

Group	Protease	Treatment	Fatty acids(%)	Linoleic acid (%)
Control	Pancreatin + pepsin+ bile extract	Maltodextrin	100.0±20.0	100.0±28.9
AstraGin®	Pancreatin + pepsin+ bile extract	**AstraGin®**	200.0±20.0**	184.9±33.1**
AstraZyme® Lipid + AstraGin®	**AstraZyme® Lipid**+ pepsin+ bile extract	**AstraGin®**	220.0±40.0**	209.6±45.8**

** p<0.01, when compared to control group

3.Objective

AstraGin® has been validated and demonstrated to enhance the cellular absorption of amino acids, vitamins, and glucose in NuLiv Science's ***In vitro*** and ***In vivo*** studies. Details of the studies are presented in the AstraGin® product dossier.

The purpose of this study is to determine the percent of NUTIVA hempseed oil that is broken down to fatty acids by pancreatin + pepsin+ bile extract or AstraZyme® Lipid + pepsin + bile extract and the effect of AstraGin® on the absorption of fatty acids and linoleic acid in human small intestine Caco-2 cells.

4. Materials & Methods

Cell Culture

The Caco-2 cell line was obtained from ATCC (Philadelphia, PA,USA). The Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum(Gibco Life Technology), nonessential amino acids, L-glutamine and penicillin/streptomycin. The Caco-2 cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. The cells used in the experiments were between passages 10 and 20. Caco-2 cells were subcultured weekly by trypsin and were seeded at a ratio of 1:3 upon reaching 80% confluence. The culture medium was changed every 2–3 days. For the transport experiments, the cells were seeded at a density of $9x10^5$ cells/cm^2 in 6-well filter support inserts with polyethylene membranes (0.4 μm pore size, 24 mm diameter, 4.67 cm^2 growth surface areas; Costar, Corning Inc., Corning, NY). The monolayers reached confluence in 3 days after seeding, and the cells were differentiated for at least an additional 14 days prior to the transepithelial transport experiments. The integrity of the Caco-2 cell monolayers and the tight junctions were monitored before every experiment by determining the transepithelial electrical resistance (TEER) measurements using an epithelial Volt-Ohm Meter (Millicell ERS-2, Millipore, Bedford, MA). Only the Caco-2 monolayers with TEER values higher than 700Ω • cm^2 were used for the experiments.

Lipid micellar preparation

200±0.02 mg of hemp oil (64.97%a-Linolenic acid, 16.16%a-Linoleic acid and 18.88% Oleic acid, obtained from Barlean) were mixed with 32 ml of 0.9% NaCl (30 s at 6000 rpm), and 2.5 ml of artificial saliva was added. The samples were incubated for 10 min in a shaking water bath at 37°C. pH was then adjusted to 4±0.02 with about 500 μl of 1 M HCl. Then 2 ml of porcine pepsin (40 mg/ml in 0.1 M HCl) was added, and the mixture was incubated at 37°C in a shaking water bath for 30 min to mimic the gastric phase of digestion. The pH of the partially digested mixture was raised to 6±0.02 by adding 800 μl of 0.9M sodium bicarbonate pH 9–10. Then, 9 ml of a mixture of porcine bile extract and pancreatin (containing 3 mg/ml pancreatin and 12 mg/ml bile extract in 100 mM trisodium citrate, pH 6.0) and 4 ml of porcine bile extract at 0.1 g/ml were added. In another digestive group, pancreatin is replaced with AstraZyme® Lipid. Samples were further incubated in a shaking water bath at 37°C for 60 min to mimic the duodenal phase of

digestion. The aqueous fraction containing the mixed micelles that were produced upon in vitro digestion was separated from oil droplets by centrifugation (3600 rpm for 1 h, 4°C), then collected and sequentially passed through a 0.8-μm and a 0.22-μm filter in order to obtain a clear solution of mixed micelles. The mixed micelles were diluted in PBS-GSM medium (1:8 to 1:10, v:v) for transport study.

Transepithelial transport studies

After TEER measurement, the differentiated Caco-2 monolayers were gently rinsed twice with phosphate-buffered saline (PBS, pH 7.4) supplemented with 25 mM glucose, 10 μM $CaC1_2$ and 1 mM $MgC1_2$ (PBS-GCM). The transport experiment was initiated by replacing the incubation solution on the apical side with diluted mixed micelles. The transwells were incubated at 37℃ for 30 min and the basolateral medium were sampled at the designated time intervals. The end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω • cm^2.Results are expressed as the initial rate of total fatty acids transport across (nanomoles per minute) across the Caco-2 monolayers in mean ± SD (*n* = 3-5). Differences between means of groups were assessed by the *t*-test.

Total fatty acids assay

The fatty acids contents were assayed by abcam 65341 free fatty acids quantification kit according to the manufacturer's protocol.

Linoleic acid assay

The linoleic acids contents were assayed by BG human conjugated linoleic acid ELISA kit according to the manufacturer's protocol.

5. Results

5.1. Composition of NUTIVA hemp seed oil

Hemp seed oil	(mg/15 mL)	%
α-Linoleic acid(Ω-6)	7000	57.1
α-Linolenic acid(Ω-3)	2500	20.4
Omega 9	2000	16.3
γ-linolenic acid(Ω-6)	500	4.1
Stearidonic acid(Ω-3)	250	2.0
Total omegas	12250	100

Hemp oil has the nature's ideal 3:1 Omega-6 to Omega-3 ratio. Hemp has valuable Super Omega-3 (SDA) and Super Omega-6 (GLA), which can help the body metabolizes fat.

5.2. AstraGin® on the absorption of NUTIVA hemp seed oil derived fatty acids and linoleic acid in Caco-2 cells after 24 hour pre-treatment with AstraGin®

Table 2. Analysis of the total quantity of fatty acids and linoleic acid absorbed in Caco-2 cells in 20 minutes. Percent of fatty acids and linoleic acid absorption in NUTIVA hempseed oil digested with pancreatin + pepsin+ bile extract and AstraZyme® Lipid+ pepsin + bile extract. Treated hemp oil with pancreatin + pepsin+ bile extract was used as the control.

Group	Protease	Treatment	Fatty acid (%)	Linoleic acid (%)
Control	Pancreatin + pepsin+ bile extract	Maltodextrin	100.0±3.4	100.0±3.3
AstraGin	Pancreatin + pepsin+ bile extract	**AstraGin®**	128.1±2.8**	141.3±6.7***
AstraZyme® Lipid + AstraGin®	**AstraZyme® Lipid** + pepsin+ bile extract	**AstraGin®**	137.6±2.5**,##	155.4±3.8***,###

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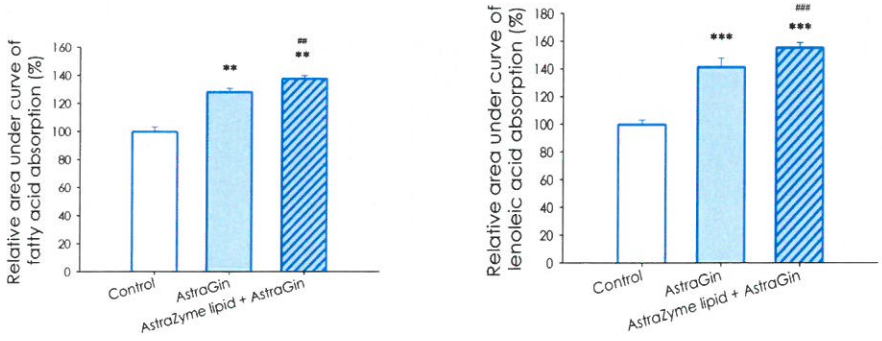

Figure 1. AstraGin® on the absorption of fatty acids and linoleic acid in Caco-2 cells after 24 hour pre-treatment with AstraGin®.

As in figure1, figure3 and figure4 (in supplement), AstraGin® was added in differentiated Caco-2 cells for 24 hour prior to the absorption studies. AstraGin® increased the absorption of total fatty acids and linoleic acid derived from NUTIVA hemp oil in the observed time. There was a maximum absorption of total fatty acids and linoleic acid In the AstraZyme® Lipid + AstraGin® group. This may be due to AstraZyme® Lipid's ability to break down more hemp oil into fatty acids.

5.3. AstraGin® on the initial transport rate of NUTIVA hemp seed oil derived fatty acids and linoleic acid in Caco-2 cells after 24 hour pre-treatment with AstraGin®

Table 2. Analysis of the initial transport rate of fatty acids and linoleic acid absorbed in Caco-2 cells in 5 minutes. Percent of fatty acids and linoleic acid absorption in NUTIVA hempseed oil digested with pancreatin + pepsin+ bile extract and AstraZyme® Lipid + pepsin + bile extract. Treated hemp oil with pancreatin + pepsin+ bile extract was used as the control.

Group	Protease	Treatment	Fatty acid (%)	Linoleic acid (%)
Control	Pancreatin + pepsin+ bile extract	Maltodextrin	100.0±20.0	100.0±28.9
AstraGin	Pancreatin + pepsin+ bile extract	**AstraGin®**	200.0±20.0**	184.9±33.1**
AstraZyme® Lipid + AstraGin®	**AstraZyme® Lipid**+ pepsin+ bile extract	**AstraGin®**	220.0±40.0**	209.6±45.8**

** $p<0.01$, when compared to control group

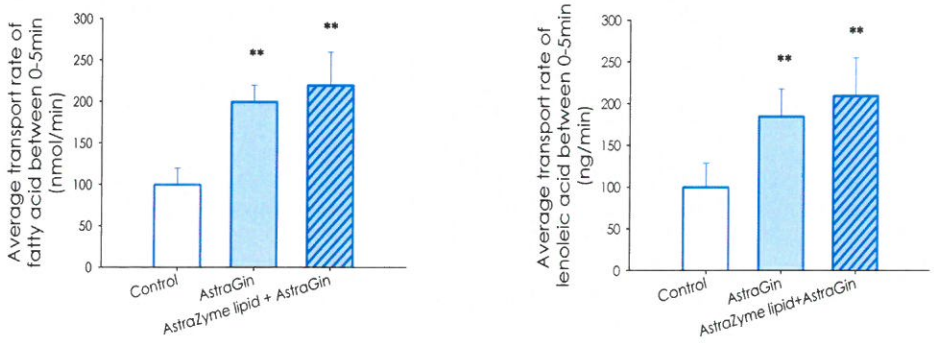

Figure 2. AstraGin® on the transport rate of fatty acids and linoleic acid in Caco-2 cells after 24 hour pre-treatment with AstraGin®.

As in Figure2, Figure3 and Figure 4 (in supplement), AstraGin® was added in differentiated Caco-2 cells for 24h prior to the absorption studies, AstraGin® increased the transport rate of fatty acids and linoleic acid derived from NUTIVA hemp oil in the observed time. AstraZyme® Lipid + AstraGin® group had the maximum transport rate of total fatty acids and linoleic acid. This again may be due to AstraZyme® Lipid's ability to break down more hemp oil to fatty acids.

6. Discussion

Non-drug varieties of Cannabis sativa L., collectively named as "hemp", have been an interesting source of food, fiber, and medicine for thousands of years. Hempseed

has been documented as a folk source of food throughout recorded history. Raw, cooked, or roasted, and hempseed oil (HSO) has been used as a food/medicine in China for at least 3000 years. Hempseed has high levels of vitamins A, C, and E, minerals, and β-carotene. It contains 20–25% protein, 20–30% carbohydrates, 25–35% oil, 10–15% insoluble fiber, and a rich set of minerals. The ever-increasing demand for vegetable oils has made it essential to characterize additional vegetable oil through innovative uses of its components. The exceptional fatty acid profile in Nutiva hempseed oil offers a rich source of the two essential fatty acids (EFAs); omega-3 alpha-linolenic acid (ALA 20%) and omega-6 linoleic acid (LA 57%), in addition to significant amounts of gamma linolenic acid (GLA 4%) and stearidonic acid (SDA 2%). We can't make the EFAs ourselves, so we have to get them from our daily diet. The EFAs are needed to produce many important chemicals in our bodies, including optimal nerve functions throughout the brain and central nervous system. It is considered to be one of the few seed oils that contain about 80% polyunsaturated fatty acids in a perfect 3:1 ratio ofOmega-6 to Omega-3, which is suggested as optimal for human nutrition. Hemp seed oil shows excellent oxidative stability suggesting the possible presence of phenolic compounds that act as antioxidants in the cold-pressed seed oil. Cannabidiol (CBD) has been found to be present in hemp seed oil as well. Although not explicitly produced within the seed, traces of cannabinoid contamination have been reported to result from the pressing of the oil. Reports of cannabinoid contamination have been focused primarily on delta-9-tetrahydrocannabinol (THC) with THC levels in oil reported at up to 50 ppm. The production and storage of both CBD and THC occur in the glandular structures of the plant and the concentrations of CBD are typically much higher than THC in most fiber and oil varieties of hemp. Therefore, it can be assumed that the concentration of CBD as a contaminant in the oil would be greater than the concentration of THC which has been reported in some literature. The presence of CBD is significant because it has documented anticonvulsive, anti-epileptic, and antimicrobial properties. Although the levels of CBD within the oil are typically small, many health benefits may still be gained from its presence.

HSO, in addition to its nutritional value, has demonstrated positive health benefits, including lipid metabolism, cardiovascular health, immunomodulatory effects, and dermatological diseases. The high amounts of α-linolenic acid may have favorable nutritional implications and beneficial physiological effects on the prevention of coronary heart disease and cancer. The presence of γ-linolenic acid provides it with a high pharmaceutical value for degenerative chronic diseases. The oil was characterized by a high polyunsaturated/saturated (P/S) ratio, which is regarded

favorably for the reduction of serum cholesterol and atherosclerosis and prevention of heart diseases. Higher plants are able to produce the essential n-6 and n-3 PUFA: linoleic acid and α-linolenic acid respectively. In contrast, animals and humans have no capacity to synthesize LNA and ALA from the precursor oleic acid due to the lack of desaturases. LNA and ALA are substrates for endogenous elongation and desaturation processes and share the same enzyme systems to produce long-chain PUFA (LC-PUFA) such as arachidonic acid and eicosapentaenoic acid, respectively.

Hemp oil extracted from hemp seeds has become an important source of edible oil for human nutrition. Besides some health effect ions, it can be more easily emulsified by emulsifiers than other frequently used oils due to high intersolubility with water. In our studies, we can observe that the uptake time for hemp oil is very quick; especially as it reaches maximum levels almost after 5 minutes in all groups. The hemp seed oil and its emulsions are formed by mimicking the gastrointestinal digestion. The easily emulsified proprietary of hemp oil makes it a valuable nutritional composition for many health benefits.

One in vivo study has demonstrated that kinetic analysis of the uptake of the three fatty acids, linoleic acid, alpha-linolenic acid and arachidonic acid indicates saturable kinetics of first order ligand binding, suggesting that, EFA absorption is facilitated by one or more carrier or binding molecules. Such transport mechanism most likely involve the 40-kDa brush border fatty acid binding protein, which has a higher binding affinity for longer chain fatty acids. Higher uptake affinities for longer chain and more unsaturated fatty acids have also been demonstrated in other tissues. In this study, AstraGin® also displays its ability to enhance total fatty acids absorption. Notably, AstraGin® increases linoleic acid absorption markedly greater than total fatty acids. AstraGin® increases more fatty acids absorption when hemp oils are digested by pancreatin+pepsin+bile extract and AstraZyme® Lipid. In the study, we verified that AstraZyme® Lipid helped more hemp oil hydrolysis into fatty acids led to more fatty acids absorption by AstraGin®. There are statistical difference between AstraZyme® Lipid+AstraGin® and AstraGin® groups. Total quantity of total fatty acids and linoleic acids are increased by 9-14% when compared to AstraGin® group. The initial transport rates between 0-5 min are elevated by 15-20% when compared to AstraGin® group. This extra enhancement should be due to AstraZyme® Lipid's efficient hydrolytic work. Although we have not studied any mechanisms about fatty acid transporters, we think increasing total fatty acids and linoleic acid absorption by AstraGin® is associated with fatty acid transporters expression. Based on literature finding and our proof, AstraGin® increases omega-7 and total fatty acids absorption in flax oil and fish oil, AstraGin® increases the absorption of many nutrients through their specific transporters, these evidences

support that AstraGin® increases total fatty acids and linoleic acid absorption through relative transporters.

Gut microbial metabolites of polyunsaturated fatty acids have attracted much attention because of their various physiological properties. *Lactobacillus plantarum* has the ability to convert linoleic acid to oleic acid via 10-hydroxy-*cis*-12-octadecenoic acid(HYA), 10-oxo-*cis*-12-octadecenoic acid (KetoA), 10-oxo*trans*-11-octadecenoic acids (KetoC), 10-oxo-octadecanoicacid (KetoB), and 10-hydroxyoctadecanoic acid (HYB). A gut microbial metabolite of linoleic acid, 10-hydroxy-*cis*-12-octadecenoic acid (HYA), ameliorates intestinal epithelial barrier impairments by GPR40-MEK-ERK pathway. The G-protein-coupled receptor 40 (GPR40), also known as free fatty acid receptor 1, has emerged as an important component in the fatty acid augmentation of insulin secretion. HYA-induced GPR40 signaling contributes to the intestinal homeostasis. Various fatty acids, such as long-chain fatty acids (for example, linoleic acid, linolenic acid, and oleic acid) and middle chain fatty acids (capric acid and lauric acid), are reported to be endogenous ligands for GPR40. Among them, linoleic acids have the highest affinity for GPR40. It has demonstrated that HYA attenuates epithelial barrier impairment in Caco-2 cells and ameliorates intestinal inflammation in DSS-induced colitis in mice. In our in vivo studies, it has been demonstrated that AstraGin® reduced ulceration in epithelial cell membrane, increased the brush border definition and clarity, and reduced edema in the sub musical layers. In this study, AstraZyme® Lipid + AstraGin® increased linoleic acid absorption. Combination of hemp oil with AstraZyme® Lipid + AstraGin® can be effective in supporting gut immunity and anti-inflammation.

The reported health benefits of hemp seed oil, and especially the essential fatty acids, are well documented. When diets are supplemented with omega-6 and omega-3 PUFA in the proper 3:1 ratio, numerous benefits to health are achieved, including but not limited to greater resistance to cancer, inflammation, and blood clotting. A general increase in metabolism and lowering of overall blood cholesterol levels has also been observed. In addition to all of these positive health benefits associated with the use of hemp oil, there seems to be a complete lack of negative effects from its consumption. To date, there has been no reported case of toxicity from the ingestion of hemp seed oil. Toxicity has also not been observed with any of the other constituents that were found as contaminants, which are primarily the cannabinoids. One reason for the lack of negative side effects from excessive ingestion of hemp oil is specifically related to the ratio of LA:LNA. Because most oils do not contain the optimum ratio of omega-6 andomega-3 PUFA, they tend to

promote the accumulation of metabolic intermediates that in turn hinder fatty acid metabolism. The properly balanced hemp seed oil does not promote an over-accumulation of certain metabolic products and all fatty acid metabolic pathways have the necessary intermediates to work efficiently regardless of the quantities consumed.

7. References

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8. Supplement

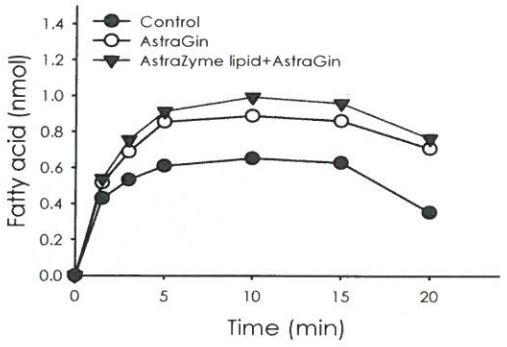

Figure3. Effect of AstraGin® on total fatty acids absorption in Caco-2 cells between 0-20 min after 24h treatment of AstraGin®. NUTIVA hemp oil are digested by digestive enzymes, pancreatin + pepsin+ bile extractor AstraZyme® Lipid + pepsin + bile extract for 2h, and then added into Caco-2 for amino acids study.

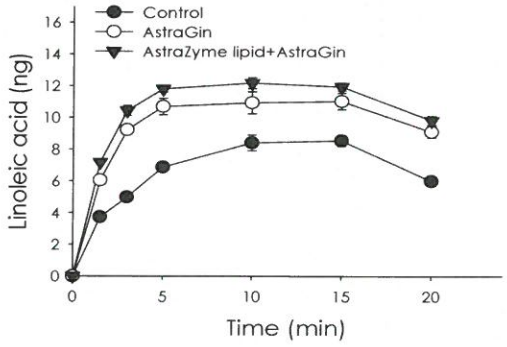

Figure4. Effect of AstraGin® on linoleic acid absorption in Caco-2 cells between 0-20 min after 24h treatment. NUTIVA hemp oil are digested by digestive enzymes, pancreatin + pepsin+ bile extractor AstraZyme® Lipid+ pepsin + bile extract for 2h, and then added into Caco-2 for amino acids study.

AstraGin® is a registered trademark of NuLiv Science USA, Inc.

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