CERA-Q™

Silk Protein Hydrolysate

Brain Effects & Human Clinical Studies

White Paper

NOVEL INGREDIENTS

72 Deforest Avenue, East Hanover NJ 07936
P: 866-668-3550 F: 973-808-5959

CERA-Q™
SILKWORM COCOON FIBROIN PROTEIN HYDROLYSATE BRAIN EFFECTS & HUMAN CLINICAL STUDIES SYNOPSIS

Introduction:

Cera-Q is a specially prepared, proprietary enzymatic hydrolysate extract of fibroin protein from silkworm (*Bombyx mori*) cocoons intended to support brain health and mental functions. Cera-Q is unusual for a brain-specific nutrient in that it is not derived from herbs, not a lipid, not a cellular metabolite, not a vitamin or mineral, but is a specific collection of bioactive peptides (short sequences of amino acids). Historically, silk protein hydrolysates have been consumed as food and in traditional Asian medicine for its purported health benefits, and its use as a prepared nutraceutical spawned from biomedical research on the hydrolysate.

Characterization of Cera-Q

It is important to understand and appreciate the benefits seen in human clinical studies from Cera-Q are derived from the unusual and specific structure of silk fibroin protein found in silkworm cocoons. Thus, a brief familiarization with the protein structure of silkworm cocoon fibroin protein is presented here.

Silkworm Cocoon Protein – Fibroin

Cera-Q is prepared from cocoons of the silkworm moth, *Bombyx mori*. The larva is not used for preparation of Cera-Q, only the cocoon. Silkworm cocoons are the source for silk used to make fabric, sutures, biomaterials and also, Cera-Q. Silkworms are cultivated as for silk production, and fed mulberry tree leaves from devoted plantations in a protected environment. Silkworms generate their cocoon from a single thread of silk about 300-1000 meters length in two days, secreted from specialized glands in the caterpillar larva (Mondal et al., 2007). Silkworm cocoon threads are similar to spider web threads, but usually thicker (about 10 microns).

Silkworm cocoons are composed almost entirely of protein (see Table 1). Silkworm cocoon protein is mostly fibroin – the tough, resilient inner core protein of the thread (see Figure 1). Fibroin is a large protein – 391 kDa as determined by sequencing its gene (Hu et al., 2006).

Table 1: Composition of silkworm cocoon (http://www.fao.org/docrep/x2099e/x2099e03.htm)

Component	%
Fibroin protein	72-81
Sericin protein	19-28
Fat & wax	0.8-1.0
Ash & coloring matter	1.0-1.4

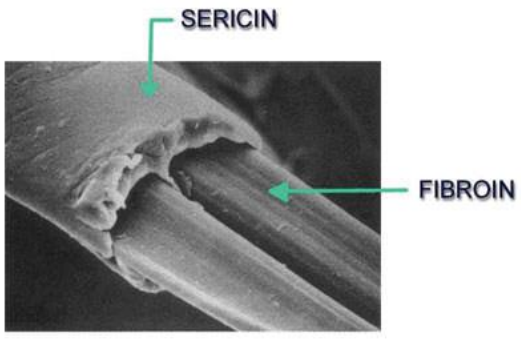

Figure 1: Electron micrograph of a silk strand showing outer sericin coating and inner core of fibroin.

In silk, fibroin proteins are interconnected into a continuous, polymeric, mesh network that forms a single silk strand (Gosline et al., 1999; Hu et al., 2006). Silkworm fibroin has a very specific sequence of regions or blocks that account for its unusual abilities (Gosline et al., 1999; Hu et al., 2006). Repeated blocks of hydrophobic poly-glycine (24-35 residues) and poly-alanine (8-10 residues) sequences form a common protein structure called beta sheets. Sheets are arranged to be adjacent when folded, forming molecular-level attractions from hydrogen bonding and van der Waals forces that produce a crystalline, polymer mesh network (similar to a laminate) accounting for tensile strength, stiffness and toughness (Gosline 1999; Hu et al., 2006; Krejchi et al., 1997; Vepari & Kaplan 2007). Think of the beta sheet linkages as a form of "Molecular Velcro®."

This unusual characteristic is important for the bioactivity of fibroin peptides, and will be discussed later in this document. To achieve this structural feat, fibroin's amino acid composition is unusual, consisting mostly of glycine and alanine residues (about 75%) (see Table 2). Other amino acids occupy key positions in the protein strand sequence and account for how fibroin folds into a single, continuous, long strand of silk protein with macroscopic physical characteristics.

Amino Acid	g/100 g protein
Alanine	32.4
Arginine	0.9
Aspartate	1.9
Cysteine	0.1
Glutamate	1.7
Glycine	42.8
Histidine	0.3
Isoleucine	0.9
Leucine	0.7
Lysine	0.5
Methionine	0.2
Phenylalanine	1.2
Proline	0.6
Serine	14.7
Threonine	1.2
Tryptophan	0.5
Tyrosine	11.8
Valine	3.0
Total	115.4

Table 2: Amino Acid Composition of Silkworm Fibroin (http://www.fao.org/docrep/x2099e/x2099e03.htm)

The other major protein in silk, sericin, is the sticky outer protein sheath for silkworm cocoon threads. It is removed during processing of CERA-Q and thus does not contribute to functionality of CERA-Q. There are several chaperone proteins (light chain fibroin & P25) that guide formation of the silk thread out of the glands, maintain the shape of the silk thread and provide for attachment of color compounds. (Zhang et al., 2006) These are also washed away with sericin and are not part of CERA-Q.

CERA-Q Production

CERA-Q is produced by a proprietary process that breaks down silk cocoons into smaller peptides (strings of amino acids that are too small to be a protein). Figure 2 illustrates the steps in CERA-Q production. Silkworm cocoons (minus the worm) are washed and the outer sericin protein is removed by basic surfactant washing. The remaining fibroin protein is heated with calcium chloride in order to relax and open up the protein sheets so that enzymes can access the peptide chains. After removal of the calcium salt by filtration, a proprietary vegetarian protease is added that digests fibroin into smaller peptides. This step is critical for obtaining a peptide hydrolysate with batch-to-batch consistency of peptide identity, molecular weight and bioactivity. Proteolysis is stopped by brief heating, and the resulting liquid is freeze-dried or spray-dried.

A molecular weight range of 500-5000 daltons selects for a particular set of peptides that emphasize the alanine/glycine-rich beta sheet structural sequences of fibroin (Kang et al., 2013).

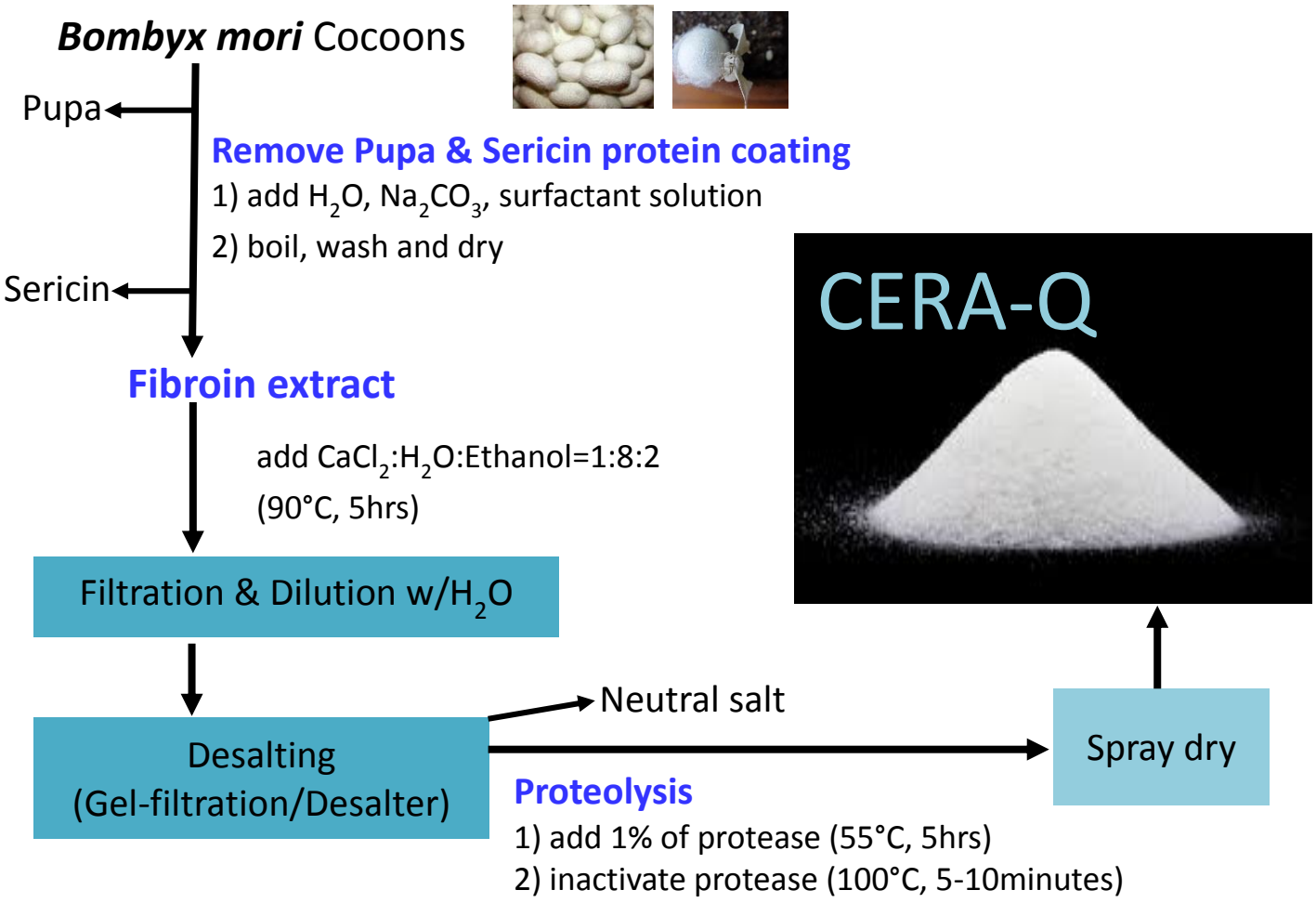

Figure 2: Method of preparation for CERA-Q

CERA-Q Characteristics

CERA-Q is a white to yellowish-brown powder with specifications of >85% protein, <10% moisture and acceptable ranges of heavy metals and microbial counts. CERA-Q is stable for at least two years when stored properly. A 1% solution has a pH between 5.5-7.5, and a semi-sweet taste similar to glycine. CERA-Q has good solubility in water and aqueous solutions, since it is soluble during production. CERA-Q powder particle sizes can be adjusted to customer specifications. CERA-Q is a free-flowing powder amenable to tablets, capsules and liquids production.

CERA-Q Regulatory Status

GRAS Status?

CERA-Q is an enzymatic protein hydrolysate. As such, it falls under the US FDA's Select Committee On GRAS Substances (SCOGS) 37b from 1972-1980 (http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/SCOGS/ucm261285.htm). SCOGS 37b states: "…the Select Committee concludes that: There is no evidence in the available information on acid hydrolyzed proteins, ***enzymatically hydrolyzed protein***, yeast autolysates, and soy sauces, that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when they are used as flavoring agents at levels that are now current or that might reasonably be expected in the future." [Emphasis mine] From the SCOGS Opinion, flavoring refers to a usage amount of less than 3mg/kg/d of hydrolysates. This would be 210 mg daily for a reference human (70 kg). The typical dose of CERA-Q for efficacy is 100-200 mg per serving.

FDA lists the full Report for SCOGS 37b Opinion as "Enzymatically hydrolyzed protein" under NTIS (National Technical Information Service) Accession Number PB283440 entitled "Evaluation of the Health Aspects of Protein Hydrolyzates as Food Ingredients." This 40-page report from 1978 does not distinguish which protein is used for the hydrolysate, and considers the status of all enzymatically

hydrolyzed proteins as GRAS. This fact is not mentioned in the freely accessible SCOGS 37b Opinion on the FDA website. However, silk fibroin protein hydrolysate is not specifically mentioned, and application of the FDA SCOGS 37b GRAS opinion needs to be determined with legal counsel for each application. In other words, we are not advocating listing CERA-Q as FDA-approved GRAS without legal counsel review. Also, CERA-Q is not self-affirmed GRAS as no submission has been made.

The Department of Food Standards of the Korea Food and Drug Administration (KFDA) issued a response in 2010 (Inquiry No. 43861) to the manufacturers of CERA-Q stating that "…Silk peptide made from silk cocoon by hydrolysis with acid or enzyme can be used as food." In 2014, the Minister of Ministry of Food and Drug Safety of the KFDA issued a Certificate of Raw Material for Functional Foods (2014-24).

NDI Opinion

Additionally, an opinion letter from the office of Anthony L. Young, Esq. of Kleinfeld, Kaplan & Becker, LLP concluded that CERA-Q "…is not a new dietary ingredient requiring premarket notification to the Food and Drug Administration" under 21 USC 350b(a). CERA-Q meets the definition of a dietary supplement ingredient that has been in the food supply as an article used for food in a form in which the food has not been chemically altered.

CERA-Q has been a food in common use with no need for New Dietary Ingredient notification, approved use in Korea as a food and functional food, SCOGS GRAS status and without safety concerns at the dosages studied (200-400 mg/day).

Structure-Function Claims for Brain Health

Based solely on randomized, controlled trials (RCTs) on normal, healthy individuals, dietary supplement product claims based on these CERA-Q study results can be considered as Structure-Function Claims as defined by the Dietary Supplement Health & Education Act of 1994 (http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/SignificantAmendmentstotheFDCAct/ucm148003.htm) and as refined by the FDA Final Ruling from 2000 on Structure-Function claims (Department of Health & Human Services 2000).

Furthermore, these RCTs on CERA-Q used the same unaltered material and same doses as available for dietary supplement products. Thus, claims based on these human studies (at doses of 200-400 mg/day) comply with the Advertising Guidance for Dietary Supplements published by the US Federal Trade Commission (FTC) in 2001 (Bureau of Consumer Protection 2001; https://www.ftc.gov/tips-advice/business-center/guidance/dietary-supplements-advertising-guide-industry), as well as similar updated guidance from the FDA (Office of Nutrition, Labeling, and Dietary Supplements 2008).

CERA-Q & Brain Health

Brain health encompasses both organ-specific (attention, concentration, cognition, executive functions, focus, intelligence, neuronal activity, spatial orientation) and common (cellular bioenergetics, cellular metabolism, gene expression, electrical potential, neurotransmitters, vascular function) properties that relate to nutrition.

Knowledge of potential mechanisms of action for CERA-Q peptides from preclinical studies (detailed later in this document) included beneficial effects on amyloid plaque and brain neurotransmitter levels linked to brain function. Therefore, coupled with known safety, it was logical to conduct human clinical studies on memory, cognition and other brain functions.

CERA-Q Human Clinical Study Results

There are 9 original human clinical studies on CERA-Q and mental functions published in 11 peer-reviewed reports and one US patent application (Chae et al., 2004; Kang et al., 2013; Kim et al., 2004; Kim et al., 2005; Kim et al., 2009a; Kim et al., 2009c; Kim et al., 2010b; Kim et al., 2011; Lee et al., 2004a; Lee et al., 2004b; Lee et al., 2004c; Lee et al., 2005). All used normal, healthy, Korean subjects. Ages studied ranged from children to high school students to adults to seniors. Both genders were evenly represented in all studies. Thus, results are relevant to normal, healthy populations and are not extrapolated from a disease treatment setting or gender-specific. Doses studied were 200 or 400 mg daily, divided into two equal doses per day (100 mg b.i.d. or 200 mg b.i.d.), except for one study that used 10 mg CERA-Q daily (Kim et al., 2009a). Duration of studies was 3-4 weeks except for one study on children that lasted 16 weeks. All but two studies were randomized, double-blind, placebo-controlled designs, with two or three parallel groups. Therefore, CERA-Q human studies on brain health are regarded as high-quality, and fit the highest level of evidence for claim support as stated by both the FDA and FTC.

Appendix A contains further details on the assessment tools used in human studies of brain function with CERA-Q.

CERA-Q Human Clinical Study Results for Brain Health

CERA-Q and Brain Health in Children

Two randomized, double-blind, placebo-controlled studies on children administered CERA-Q have been published (Kim et al., 2009c; Kim et al., 2010b).

Kim et al., 2009c

Kunwoo Kim and coworkers from Chung-Ang University, Dankook University, Seoul National University, University of Florida and University of Ulsan reported on the effects of CERA-Q on mental functions of children (Kim et al., 2009c). Normal, healthy Korean schoolchildren aged 9.9 ± 1.1 years were divided into two groups of 23 subjects each and given placebo or 400 mg CERA-Q/day (200 mg b.i.d.) for 16 weeks. There were 27 girls and 19 boys, and they started with an average IQ of 116.7 ± 9.8. Baseline assessment with the K-SADS-PL-K instrument determined that all subjects were normal, of high average intelligence and without psychiatric diseases or conditions.

Both CTT-1 and CTT-2 tests were solved significantly more quickly and with fewer errors in the CERA-Q group, but not the placebo group. For CTT-2, the improvement in speed to complete the task was 23% vs. 9.8% for placebo (see Table 3).

Table 3: Results of the CTT-2 speed of completion between Placebo and CERA-Q Study Groups

	CTT-2 Time (seconds)			
	Placebo (n=23)		CERA-Q (n=23)	
	Baseline	16 weeks	Baseline	16 weeks
Mean (SD)	82.6 (24.8)	74.5 (20.1)	96.7 (30.6)	74.8 (16.3)
P value	>0.05		<0.05	

Repeated measures of multivariate analysis of covariance were used to find changes before and after administration of placebo or CERA-Q. Statistical significance defined as $P<0.05$.

Adapted from Kim et al., 2009

CERA-Q reduced errors by 28 and 43% for CTT-1 and CTT-2 tests, respectively. The authors concluded:

> "When these findings are taken together, CERA-Q has been shown to improve brain function by conducting tasks more efficiently and precisely. These measures are tightly connected with learning and memory; thus, CERA-Q enhanced the learning and memory of children."
> "In conclusion, our results clearly showed that CERA-Q improved brain function such as attention and cognitive flexibility in normal schoolchildren."

Kim et al., 2010b

Do-Hee Kim and coworkers from Chung-Ang University, Dankook University, National Academy of Agricultural Science, Seoul National University and Suheung Capsule Co. Ltd., reported on the effects of CERA-Q on mental functions of children to reproduce previous findings of efficacy and safety (Kim et al., 2010b). Normal, healthy Korean schoolchildren (n=36, 21 girls and 15 boys) aged 7-12 years (9.78 ± 1.9 years average) were divided evenly into placebo and CERA-Q (400 mg/day as 200 mg b.i.d.) groups and followed for four weeks. Baseline testing of Memory Quotient by the Rey-Kim Memory Test for Children showed both groups had similar scores that were normal.

The CERA-Q group exhibited large, highly significant improvements from baseline for short- and long-term memory, along with improved memory preservation, memory application and awareness of complex information. Placebo group was significantly improved from baseline in three of six tests. Although between-group comparison was not reported, it can be inferred from the standard deviations that CERA-Q had borderline statistical significances and very large numerical differences in test scores from placebo – 1.6x to 12x. In practice, these differences are noticeable and relevant.

Memory Quotient was improved 35% by CERA-Q and 10% by placebo – both changes were significantly improved from baseline ($P<0.001$). Immediate recall was significantly improved 41% for immediate recall of Auditory/Verbal Learning Test, and delayed recall was significantly improved 29% by CERA-Q from baseline (both $P<0.001$). Placebo group improved significantly from baseline 28% and 15% (both $P<0.001$). Improvements in the three scores from the Complex Figure Test were strongly in favor of the CERA-Q group (18 vs. 0% for Direct Copying; 54 vs. 5% for Immediate Figure Recall; 93 vs. 4% for Delayed Figure Recall). All CERA-Q differences from baseline were significant, but changes in Placebo group were not significant.

In complicated tasks requiring higher executive functions and coordination of different brain activities, CERA-Q produced large and dramatic improvements in memory and learning whereas placebo did not cause changes. A relatively short time period of four weeks was sufficient to produce improvements. In conclusion, the authors stated:

> "These results showed that long- and short-term memories were significantly improved. These results indicate that CERA-Q is a promising substance from natural resource improving learning and memory of children…"

Two RCTs with normal, healthy schoolchildren using four different assessment tools found large and significant improvements in attention, cognition, focus, learning and memory from 400 mg CERA-Q per day, with results found 4-16 weeks of intake. No adverse events were reported for CERA-Q groups. Thus, CERA-Q appears to be a safe and effective supplement to the diet of children to improve mental functions. Learning and memory are directly related to scholastic performance in children (Payton et al., 2008).

CERA-Q and Brain Health in High School and College Students

One randomized, double-blind, placebo-controlled study on high school students (Chae et al., 2004), one open-label study on college students (Kim et al., 2009a) and one open-label brain imaging study on college-age adults (Lee et al., 2004a) studied the effects of CERA-Q on mental functions and brain circulation.

Chae et al., 2004

Chae and 12 co-investigators from Chung-An University, the National Institute of Agriculture Science Technology of the Korean FDA, and Seoul National University administered 200 mg CERA-Q b.i.d. (400 mg/day, n=30) for three weeks or placebo (n=10) after baseline testing to normal, healthy high school students. Both groups also received EPA/DHA supplements during the study period (an active placebo). Assessment of mental functions was by the Rey-Kim Memory Test and also by the

memory part of the K-WAIS test (explained in more detail in Appendix A).

After three weeks, the CERA-Q group achieved significant improvements over baseline and placebo group for Memory Quotient (MQ), Learning Gradient, Memory Preservation Index (from Rey-Kim Memory test) and for K-WAIS Number-Memorizing Scores (Figure 3).

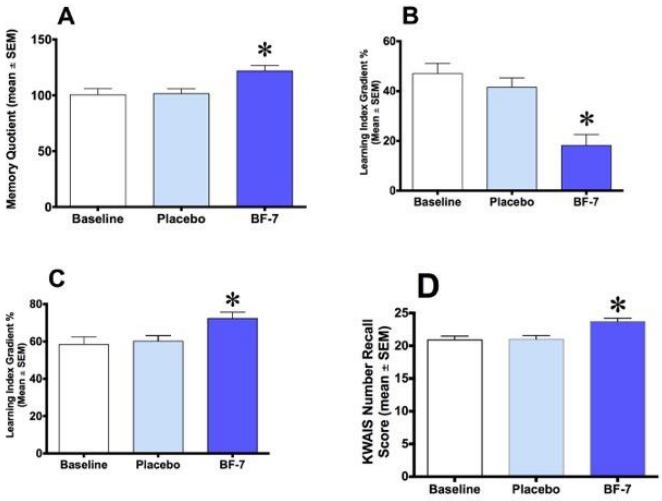

Figure 3: Results of mental function tests before and after Placebo or daily 400 mg CERA-Q for four weeks to normal, healthy high school students (adapted from Figure 5 of Chae et al., 2004). **A:** Memory Quotient scores from Rey-Kim test significantly improved by CERA-Q compared to Baseline and Placebo ($P<0.0126$ by one-way ANOVA). **B:** Learning Gradient Index % from Rey-Kim test significantly improved by CERA-Q compared to Baseline and Placebo ($P<0.0001$ by one-way ANOVA). **C:** Memory Preservation Index % from Rey-Kim test significantly improved by CERA-Q from baseline and Placebo ($P<0.0275$ by one-way ANOVA). **D:** Number Recall score from KWAIS test significantly improved by CERA-Q from Baseline and Placebo ($P<0.0015$ by one-way ANOVA).

Memory Quotient was improved by 3.5% and 22% for Placebo and CERA-Q groups, respectively, a significant difference of 6.3x. Learning Gradient Index from the Rey-Kim Memory Test was improved by 2.9% and 65.2% for Placebo and CERA-Q groups, respectively, a significant difference of 22.5x. Memory Preservation Index of the Rey-Kim Memory Test was improved by 3.1% and 23.9% for Placebo and CERA-Q groups, respectively, a significant difference of 7.7x. The K-WAIS Memory Score was improved by 0.65% and 14.4% for Placebo and CERA-Q groups, respectively, a significant difference of 22.1x.

For normal healthy high school students also receiving EPA/DHA supplements, addition of 400 mg/day of CERA-Q led to large improvements in memory and learning. The placebo group (EPA/DHA) showed only minor improvements not statistically or clinically significant. Similar to schoolchildren, a short time period of four weeks was tested. The differences between placebo and CERA-Q groups for memory improvements were large – from 6-22 times greater. The investigators stated:

> "In the case of placebo, there was no significant enhancement of learning and memory. However, with CERA-Q…it was significantly improved. …the positive findings might be arised from CERA-Q."
> "Since the CERA-Q is derived from a non-toxic natural product, it will be a preferred substance for…improving learning and memory."

Kim et al., 2009a

DH Kim and coauthors from Chung-Ang University, Konkuk University, National Academy of Agriculture science, and Seoul National University studied 30 normal, healthy university students (21 ± 1.2 years) to determine if CERA-Q in milk was able to affect brain performance (Kim et al., 2009a). Attention, learning, mathematical ability, memory and working memory were assessed by two versions of the Paced Auditory Serial Addition Test (PASAT) before and after 30 days of 10 mg CERA-Q per day in 200 mg ordinary commercial plain milk. The design appeared to be open-label, as before-after data was presented.

After 30 days, significant reductions in error rates were reported as measures of working memory, attention and mathematical abilities (Figure 4). Error rate was reduced from 1.46 to 0.42 in the Gronwall PASAT version, a 3.5x improvement in accuracy. A similar improvement was reported for the Levin PASAT version (3.7x improvement in accuracy).

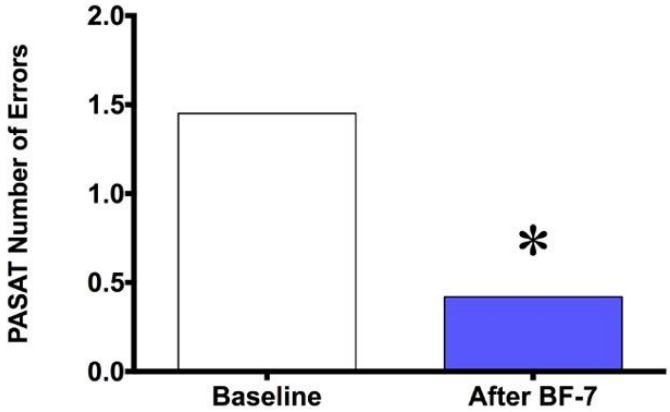

Figure 4: Improvement in error rates for the Gronwall PASAT number addition test was 3.5-fold after 30 days of 10 mg CERA-Q in milk fed to normal, healthy university students. After CERA-Q, error number was significantly reduced ($P<0.05$ by Student's paired t test) (adapted from Figure 1A of Kim et al., 2009a).

This study was primarily conducted to ascertain whether co-administration of CERA-Q with food, especially protein, would negate the ability of CERA-Q to improve brain functions. Although not placebo-controlled or crossover, this study did find large changes in brain function, suggesting that CERA-Q actions are not changed by concomitant ingestion with foods or protein. This is an important finding for relevance to real-life settings where many consumers of CERA-Q will be co-ingesting it with meals or protein. Also, product types are expanded into functional foods instead of only pills or single-ingredient liquids.

Previous studies with CERA-Q showed large changes in brain function measurements, which persuaded the investigators to not apply a controlled study design in this case. Use of a relatively harsh test (PASAT) also decreases learning effects of before-after testing, and again influenced study design. Other CERA-Q human studies have not use the PASAT. Another important distinction of this study was the very small daily dose of 10 mg, compared to 200-400 mg doses in RCTs. Unless reproduced in a controlled study using the same measurements as previous studies, a dose this low cannot be supported by the majority of available literature on CERA-Q.

Thus, another human clinical study in normal, healthy persons found improvements in brain function after 30 days of a low-dose of CERA-Q taken with milk, suggesting that CERA-Q can be ingested with or without food and still show effects.

Lee et al., 2004a

Sang-Hyung Lee and collaborators, from Chung Ang University, National Institute of Agricultural Science and Technology and Seoul National University studied four healthy, normal young adult volunteers (two women and two men, average age of 23 years), in an open-label study to assess effect of CERA-Q on intelligence. They administered 200 mg CERA-Q b.i.d. (400 mg/day) for three weeks. K-WAIS IQ test (Korean version of the Wechsler Adult Intelligence Scale) and SPECT (Single Photon Emission Computed Tomography) scans of brain blood flow were measured before and after three weeks.

K-WAIS IQ test scores before and after CERA-Q administration for three weeks were 103 (98-107) and 114 (109-122). Even though Table 1 listed a nonsignificant P value (P=0.5), this was a typographical error since the text of the original Korean language report listed this P value as P<0.05) (Bucci L, personal observation, 2015). Other statements in the Discussion and other publications also supported a statistically significant improvement in IQ from this study.

In an effort to ascertain a mechanism for the observed improvements in brain function, subjects underwent SPECT brain scans after injection with 11.1 MBq/kg technetium-99m. Technetium uptake (%) is a measure of blood circulation 30 minutes after administration. The areas of the brain that participate in learning and memory activities were visualized. Technetium uptake increased significantly from 101.1-105.4% in the parahippocampal gyrus and 95.2-104.4% in the medial temporal area (both P<0.05). Imaging also found increased consumption of glucose in these areas.

Thus, a possible mechanism of action for mental improvements seen in human subjects by CERA-Q is suggested by brain imaging data. Brain circulation and glucose uptake were increased in the areas that actuate learning and memory. The investigators concluded:

> "This means that the regions controlling cognition, memory and learning ability effectively function by administration of CERA-Q and there is an increase in blood supply and glucose consumption."

The imaging results of improved circulation correlated with K-WAIS IQ test score improvements. Although a placebo learning effect cannot be ruled out, the juxtaposition of brain blood flow improvements with mental function improvements in normal, healthy young adults using the same dose and duration of CERA-Q as seen in other successful human studies argues strongly that improved blood flow is a central mechanism for CERA-Q efficacy. Other evidence supports this mechanism, and will be presented later in this document. This small human study ties together diverse observations from in vitro, animal and human studies on brain function, circulatory system, glucose metabolism and biochemical into a cogent, plausible mechanism of action to explain the observed results.

One RCT and two open-label studies all found significant improvements in mental functions after

administration of CERA-Q to young adults (high school and college ages). A probable mechanism of action for CERA-Q (improving brain circulation and glucose uptake in areas responsible for learning and memory) was found by one open-label study. Another open-label study using a low dose of CERA-Q along with food found benefits, suggesting that CERA-Q can be taken with foods and still have efficacy for brain functions. The results are similar quantitatively to results with children taking CERA-Q, lending reproducibility, credibility and real-life relevance to using CERA-Q for brain health and performance.

CERA-Q and Brain Health in Adults

Three original studies were published in five separate reports (Kang et al., 2013; Kim et al., 2005; Lee et al., 2004b; Lee et al., 2004c; Lee et al., 2005). All were randomized, double-blind, placebo-controlled designs ranging from 31-99 subjects on a per protocol basis. All subjects were normal, healthy volunteers ranging in age from 13-70 years, representing all stages of adults. CERA-Q doses were 200 or 400 mg daily for three weeks.

Lee et al., 2004c

Subjects in this study were 66 normal, healthy Korean adults aged 42 ± 16 years (53 females, 13 males: employees, housewives, university students and visitors to religious organizations). They were given 0, 200 or 400 mg CERA-Q/day in four capsules daily (2 capsules b.i.d.) for three weeks. The K-WAIS test section of "number memorizing" (Digit Symbol Test) was administered.

Results of the K-WAIS subtest showed that the placebo group did not change, but both CERA-Q groups showed significant improvements from baseline and from placebo (Figure 5). Scores were 11.3% and 22.2% higher than baseline for 200 mg and 400 mg CERA-Q doses per day, respectively. Subgroup analysis found no differences between age or gender.

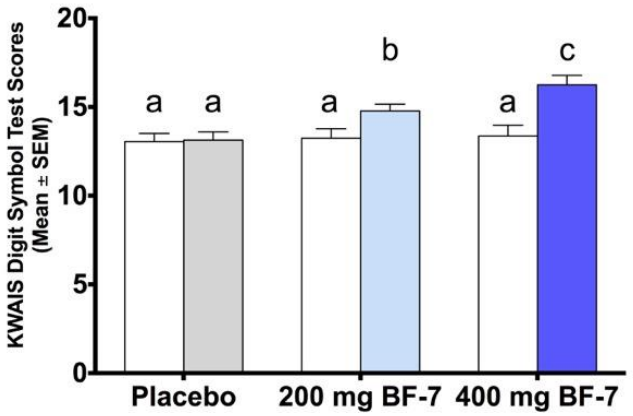

Figure 5: K-WAIS Digit Symbol test scores (mean ± SEM) before and after three weeks of Placebo or CERA-Q. Clear bars are Baseline (Before) scores, and colored bars are After scores. Bars with different letters (a,b,c) were significantly different (P<0.05) by One-Way ANOVA with Tukey's Multiple Comparison Test (between groups) or paired t test (within group). Both CERA-Q groups were significantly improved compared to the Placebo group as well as from baseline. Adapted from Figure 3 of Lee et al., 2004c.

Kim et al., 2005; Kim et al., 2011; Lee et al., 2004b; Lee et al., 2005

This randomized, double-blind, placebo-controlled study was reported in three separate reports (Kim et al., 2005; Lee et al., 2004b; Lee et al., 2005). A group of 119 volunteers representing the general Korean population was studied, but data was reported on the 99 finishers (per protocol). Similar to the population, the subjects represented a range of mental function from high level to some normal, age-related deterioration (by Rey-Kim Memory Test baseline scores). Doses of 0 (n=32), 200 mg CERA-Q (n=33, 2 capsules b.i.d.) and 400 mg CERA-Q (n=34, 2 capsules b.i.d.) were administered for three weeks.

Rey-Kim Memory Test was used to measure mental function before and after three weeks. Memory Quotient (MQ), a measure of overall memorizing ability, averaged 105 at baseline for all subjects. A dose-dependent increase in MQ scores was seen (Figure 6). The Placebo group did not change, but each CERA-Q group was significantly improved from baseline and from Placebo. The 400 mg CERA-Q dose was also significantly improved compared to the 200 mg CERA-Q dose. Percent changes in MQ from baseline were 3, 12, 21% for 0, 200, 400 mg CERA-Q, respectively. These improvements are similar to percent changes for other studied age groups (children, students).

A calculated measure of memory use with IQ (IQ/MQ score %) showed that CERA-Q significantly

improved efficiency, meaning better memory from the same level of IQ. The authors concluded:

> "Significantly increased MQ in a dose-dependent manner represented that the CERA-Q was very effective on the enhancing memory ability." (Lee et al., 2004b)

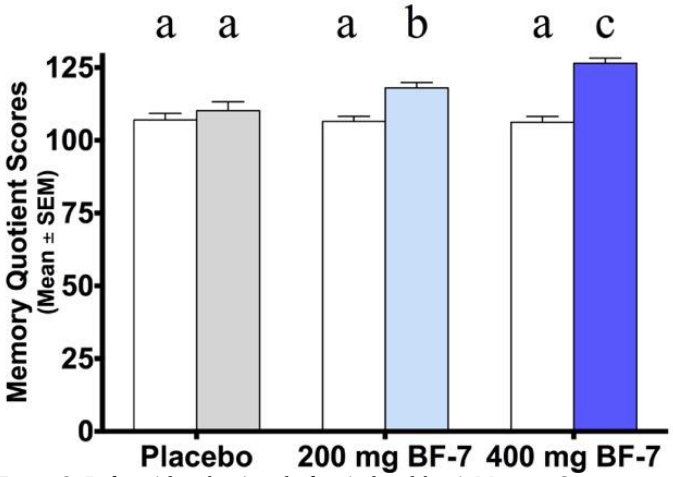

Figure 6: Before (clear bars) and after (colored bars) Memory Quotient scores from Rey-Kim Memory test after three weeks of 0, 200 or 400 mg CERA-Q daily given to normal, healthy adults. CERA-Q showed a dose-dependent, significant improvement in memorizing ability from baseline and from placebo by both doses. Bars with different letters (a,b,c) were significantly different (P<0.05) by One-Way ANOVA with Tukey's Multiple Comparison Test (between groups) or paired t test (within group). Adapted from Figure 1 of Lee et al., 2004b.

The two components of the Rey-Kim Memory test also showed significant improvements in memory functions. The RAVLT (Auditory Verbal Learning Test, described as "memory maintenance") showed a dose-dependent increase in number of words recalled (out of 15 max) and scores, with dose-dependent statistical significance for number of words recalled. The RAVLT test scores (described as "memory recall efficiency") showed significant improvements from baseline and Placebo (90 and 60 % changes from baseline for 200 mg and 400 mg CERA-Q, respectively (Figure 7).

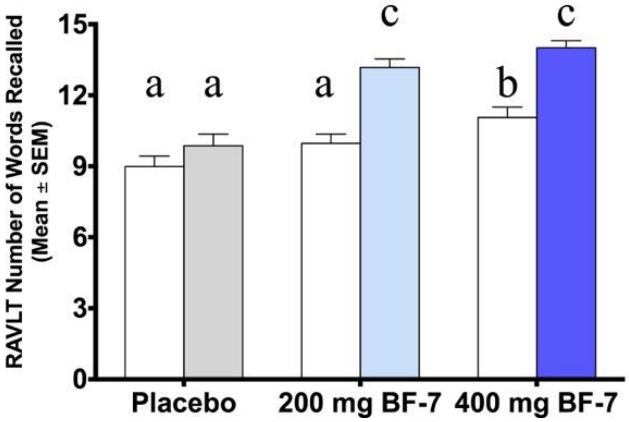

Figure 7: Changes in number of words recalled from the RAVLT section of the Rey-Kim Memory Test measuring memory maintenance in normal, healthy adults. Bars with different letters (a,b,c) were significantly different from each other (P<0.05) by One-Way ANOVA with Tukey's Multiple Comparison Test (between groups) or paired t test (within group). Adapted from Figure 4 of Lee et al., 2004b.

The visual component of the Rey-Kim Memory Test showed dose-dependent, significant improvements in scores from baseline and Placebo (Figure 8). This means that CERA-Q increased memory recall efficiency, enhanced spatial three-dimensional memory and ability to organize complex figures.

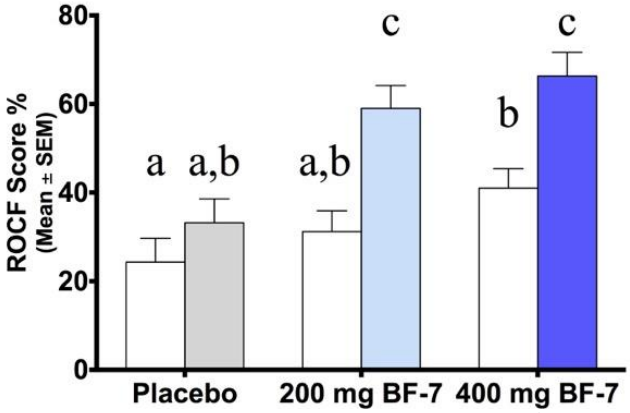

Figure 8: Rey Complex Figure Test (ROCF) score % results after three weeks of 0, 200 or 400 mg CERA-Q daily given to normal, healthy adults (mean ± SEM). Placebo group showed no change, but CERA-Q exhibited a significant improvement from baseline and Placebo at each dose. Bars with different letters (a,b,c) were significantly different (P<0.05) by One-Way ANOVA with Tukey's Multiple Comparison Test (between groups) or paired t test (within group). Adapted from Figure 5 of Lee et al., 2005.

The authors concluded:

> "Since, the CERA-Q is derived from safe natural product, together with its promising role, it is very useful material for protecting nervous system and improving learning and memory." (Kim et al., 2005; Lee et al., 2004b)
>
> "…CERA-Q is a raw material that effectively enhances brain function such as memory and cognitive function." (Lee et al., 2005)

Kang et al., 2013

Kang and coworkers from BrianOn Inc. and Inha University in Korea sought to confirm effects of a silk fibroin hydrolysate (CERA-Q) on learning and memory with the Rey-Kim Memory Test (Kang et al., 2013). 31 Normal, healthy volunteers between 13-70 years of age were divided into Placebo (n=15) and CERA-Q (n=16) groups, and administered 400 mg CERA-Q daily (200 mg b.i.d.) for three weeks.

The Memory Quotient (MQ) changed a nonsignificant 3.5% in the Placebo group and improved 22.0% in the CERA-Q group, a 6.3x

difference from Placebo and significant from baseline and Placebo group (P<0.05) (Figure 9).

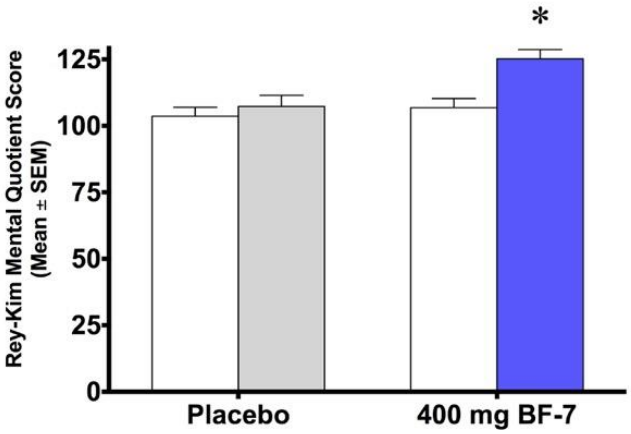

Figure 9: Memory Quotient (MQ – mean ± SEM) determined by the Rey-Kim Memory Test for 31 normal, healthy adults given Placebo or 400 mg CERA-Q daily for three weeks. MQ improved 6.3 times more than placebo by CERA-Q, a significant difference from baseline (P=0.0024) and from placebo group (P=0.0008). Adapted from Figure 2 in Kang et al., 2013.

The 22% improvement in MQ was similar to MQ improvements of 21 and 22 % from two other previous CERA-Q human studies in normal, healthy students and adults (Chae et al., 2004; Kim et al., 2005; Lee et al., 2004b; Lee et al., 2005). Thus, three separate studies of CERA-Q showed that Memory Quotient improved by about 20%.

Results of feeding three of the individual peptides found in CERA-Q to animals also had effects on memory (to be discussed later in this document as evidence that the beta peptides from CERA-Q are active agents in humans).

The authors stated:

> "These results are similar to those of previous studies [8, 14] and suggest that our SFH contains active peptides that enhance memorization ability in normal individuals."
> "In conclusion, the results of this study indicate that the enzyme hydrolysate of silk fibroin can enhance the cognitive function of normal individuals."

Thus, three separate studies in normal, healthy humans ranging in age from 13-70 years all found similar results from CERA-Q. Improvements in attention, focus, learning and memory were seen from two assessment tools in general use for decades. Finding improvements in mental functions like these in normal, healthy adults in a relatively short time period indicates remarkable results from Cera-Q.

CERA-Q and Brain Health in Seniors

One randomized, double-blind, placebo-controlled study of seniors has been published for CERA-Q (Kim et al., 2004). An unknown number of subjects in the three CERA-Q adult studies were over 60 years of age, so there is other evidence that CERA-Q may have an effect on seniors.

Kim et al., 2004

A single randomized, double-blind, placebo-controlled human study on 25 seniors (16 females, 9 men) visiting a day care center for dementia in Seoul was conducted by DK Kim and collaborators from Chung-Ang and Seoul Universities (Kim et al., 2004). Subjects were 72 ± 5.1 years average age, and not being treated for any diseases, representing a normal senior population. Subjects received either 0 or 400 mg CERA-Q per day (as 2capsules b.i.d.) for three weeks.

MMSE-K scores were unchanged in the Placebo group (25 to 25), but improved 13% in the CERA-Q group (24 to 27, P<0.05 from baseline) (see Figure 10). Improvements were made in each of the five categories of the MMSE-K test. When subjects were stratified according to degree of dementia (normal, mild dementia, severe dementia), CERA-Q had greater effects from baseline on subjects with dementia (17 and 35% improvements, P<0.05) (see Figure 11).

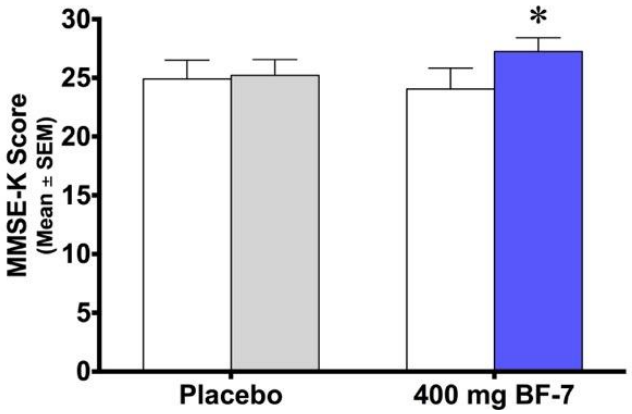

Figure 10: MMSE scores (mean ± SEM) for 25 seniors showing no change for Placebo subjects but a significant improvement from baseline in the CERA-Q group (400 mg daily for three weeks) (P<0.05). Adapted from Figure 4 of Kim et al., 2004.

The authors stated:

> "These results suggest that CERA-Q is not only effective to improve memory of high school students but also effective to improve complex cognitive function in the elderly people with dementia.
> CERA-Q is a valuable substance that is worth investing to develop an effective and safe agent that improves

learning and memory by reinforcing the cholinergic system of the central nervous system."

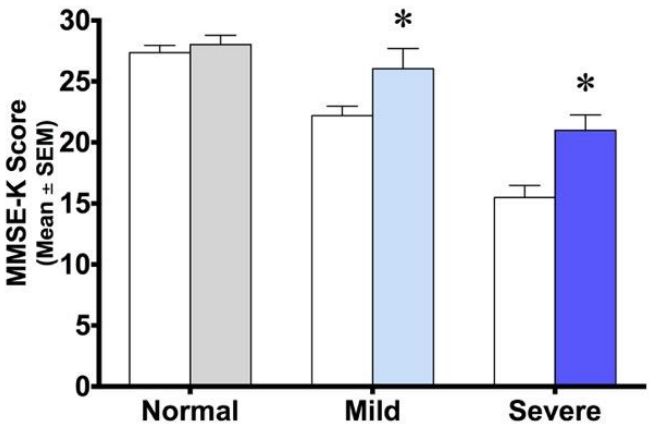

Figure 11: MMSE-K scores (mean ± SEM) of the CERA-Q group stratified according to level of memory loss showing increasing improvements from baseline as initial memory loss worsened ($P<0.05$).

Thus, in functioning seniors, CERA-Q improved cognition and memory in those with impaired functions. In this study, normal function was too close to the maximum score to show a change, unlike other measurement tools used in younger populations.

Summary of CERA-Q Human Clinical Study Results for Brain Health

In nine separate human studies over a ten-year period, CERA-Q improved mental functions of attention, cognition and memory in persons ranging in age from 9-80+. Different assessment tools appropriate for category of normal, healthy subjects were applied, and routinely showed statistically significant improvement from baseline, and often from placebo groups. Magnitude of change was from 10-20% more function, which is significant and could lead to increases in IQ (and did in one small, open label study). Duration of studies was 3-4 weeks except for 16 weeks in one study on children. Short onset of effect is important for consumers to realize a benefit and continue with supplementation rather than be discouraged waiting months for any positive feelings. Benefits were maintained for four months, which suggests that improvements should be maintained for longer periods. Improvements were reasonable and not due to stimulation or other undesirable methods. However, long-term (greater than one year) evidence is not yet available, which would be key to showing what other data suggests – that CERA-Q operates on a level that the human body is accustomed to and can utilize efficiently. Improvements seen by several assessment methods indicate a robust response to CERA-Q that cannot be dismissed as assay-dependent.

Importantly, one human study used brain imaging and found improved circulation and uptake of glucose into areas responsible for cognition and memory. As will be seen in the next section on preclinical studies, the picture of CERA-Q as a safe means to improve mental functions when needed by making normal pathways more efficient is a desirable goal for brain nutrients.

Disease states were not investigated in human studies. Further investigations for medical purposes such as strokes, dementia, Alzheimer's disease, cerebrovascular disease, Parkinson's Disease, epilepsy, Down's Syndrome, traumatic brain injury and other abnormal brain conditions can be considered.

Interaction with medications has not been specifically tested, but the apparent mechanism of action of bolstering normal cellular functions via simple peptides suggests that CERA-Q should be compatible with other modalities of improving brain function, both pharmacological and non-pharmacological.

CERA-Q Preclinical Studies for Brain Health

CERA-Q & Animal Models of Brain Health

Beta-Amyloid Protection

Kim and coworkers treated male Sprague-Dawley rats with phosphate-buffered saline (vehicle control), beta-amyloid at 2 nmol (a dose known to lower cognitive function), and beta-amyloid with 5 and 10 mg/kg CERA-Q (Kim et al., 2005). Beta-amyloid protein was injected into the hippocampal region of rat brains. CERA-Q was given orally for two weeks after beta-amyloid injection.

Beta amyloid reduced brain acetylcholine levels by 45%. Both 5 and 10 mg/kg CERA-Q per day for two

weeks similarly restored acetylcholine levels to 78 and 80% of vehicle control levels, a significant difference from beta amyloid group (P<0.05) (see Figure 12).

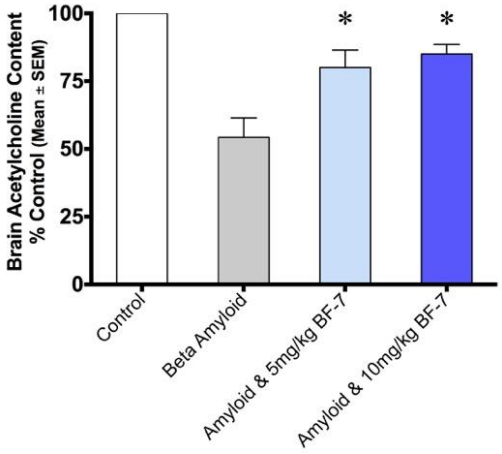

Figure 12: Whole-brain acetylcholine levels (mean ± SEM) two weeks after hippocampal injection of beta amyloid and daily oral CERA-Q at 5 and 10 mg/kg. Acetylcholine levels were reported as % of vehicle control (PBS). Beta amyloid levels were 55% of control, and CERA-Q restored acetylcholine levels to 78 and 80% of control (5 and 10 mg/kg, respectively, P<0.05 by one-way ANOVA with Tukey post-hoc comparison). Adapted from Figure 5 of Kim et al., 2005.

The authors stated:

> "It is suggested that the ability of CERA-Q helped to maintain acetylcholine levels is one mechanism for its role in enhancing brain function."

This study uncovered a potential mechanism for CERA-Q brain effects that could be explained by improved local circulation seen in humans (Lee et al., 2004a), or a more direct effect on brain cells, at a dose less than used in human clinical studies. Acetylcholine status is well accepted as vital for brain function, especially for cognition and memory (Hasselmo 2006).

Importantly, the dose extrapolated to humans was lower than that used in human clinical studies, suggesting acetylcholine maintenance as a possible mechanism of action for CERA-Q in humans.

Ischemia Protection

Lee and coworkers reported on the effect of oral CERA-Q (10 mg/kg) given after middle carotid artery occlusion for seven days (Lee et al., 2005). This dose is bioequivalent to a human dose of 112 mg daily (CDER 2005). Compared to the control group, CERA-Q reduced infarct size, and came close to normal in sham-operated animals. Neuronal cell death was significantly decreased in the CERA-Q group. Memory was assessed by the 8-arm water maze test five days after seven days of CERA-Q administration. Performance was better than control. These results indicated that oral CERA-Q showed neuroprotective effects on learning and memory from reperfusion damage, relevant to cerebral ischemia, cerebrovascular accidents and extensive surgical procedures under anesthesia.

Memory Impairment Protection

Kim and coworkers investigated effects of CERA-Q on scopolamine-induced cognition and memory loss in rats after they were trained to correctly navigate the classic water maze test (Kim et al., 2004). Once trained to find the correct platform in 15 seconds or less, rats were given placebo (5% DMSO), 1 mg/kg scopolamine (intracerebral injection) or scopolamine with 10 mg/kg oral CERA-Q. Time to reach the correct platform (latency time) was measured. After 24 hours, scopolamine-treated rats double their latency time, but those treated with scopolamine and CERA-Q showed no difference from control times. The difference between scopolamine and scopolamine with CERA-Q groups was significantly different (P<0.05) by ANOVA.

Kim and coworkers also measured memory loss with the passive avoidance test, widely used to assess working memory of rodents (Kim et al., 2004). Mice were placed in a two-compartment box with an electric grid. When the door connecting the two compartments was opened, a light illuminated the original compartment. Mice were trained to move into the dark compartment, and the time taken to move recorded as the latency period. After training, mice were given a foot shock after moving to the dark compartment. When mice were trained to stay in the lit compartment, the passive avoidance test was started. Dosing of scopolamine and CERA-Q was similar to the water maze test, but mice were used as test subjects. Control mice showed a latency time of 140 seconds, but scopolamine decreased this to 30 seconds. Adding CERA-Q with scopolamine increased latency time to 90 seconds, significantly different from the scopolamine group, but not different from the control group.

Yeo and coworkers fractionated CERA-Q peptides into low (<500 daltons), medium (500-1000 daltons) and high (1000-2000 daltons) molecular weight ranges and investigated each fraction for protection scopolamine-induced memory loss in rats (Yeo et al., 2008). Low and medium molecular weight fractions outperformed the high molecular weight fraction by partially restoring memory loss induced by scopolamine. Changes over scopolamine were 25.9% for low, 50.0% for medium and 7.9% for high ranges. Enzymatic hydrolysates of silk fibroin had twice the activity of acid hydrolysates.

Similarly, Kang and coworkers investigated effects of CERA-Q on scopolamine-induced (1 mg/kg i.p.) cognition and memory loss over an eight day period in mice (Kang et al., 2013). Both CERA-Q and three individual peptides identified in CERA-Q and chemically synthesized (GA, GY, PY) were administered (10 mg/kg i.p.), as was saline for the control group. Peptides were administered 30 minutes before scopolamine and the test was given 30 minutes later.

Step-through passive avoidance test was performed to gauge long-term memory functions. Scopolamine reduced latency time by more than half, but CERA-Q and two of the three individual peptides (GY, PY) significantly restored latency time to pre-test levels (~90%, $P<0.005$)). GA peptide increased latency time also, but was not statistically significant. These data suggest that CERA-Q, via some of its peptides, restore long-term memory impairments from scopolamine.

CERA-Q and all three peptides significantly restored scopolamine memory deficits in the Morris Water maze test ($P<0.05$), as shown by reduced latency times in the maze.

In their United States Patent Application, SS Kim and coworkers investigated effects of CERA-Q on other animal models of memory loss (Kim et al., 2011). Oral CERA-Q at 1 and 5 g/kg doses were given to rats administered 6-hydroxydopamine in one brain hemisphere to induce a model of Parkinson's Disease. These doses would be 11 and 55 grams for human equivalent dose, and are higher than those used in human clinical studies. However, duration of studies was of shorter duration, either immediate or less than one week. Behavioral effects and brain dopamine and lipid peroxide levels were measured.

Brain dopamine levels (as ratio of ipsilateral / contralateral dopamine levels) was decreased to 35% by hydroxydopamine. Oral CERA-Q at 1 and 5 g/kg doses improved dopamine ratios to 60-70% of control, significantly different from hydroxydopamine group, but still below control values.

Apomorphine-induced unilateral rotation number was greatly increased in the hydroxydopamine-treated group and reduced by ~30 to 50 % from 1 and 5 g/kg CERA-Q.

Brain lipid peroxidation was measured as malondialdehyde (MDA) levels. Hydroxydopamine increased MDA levels 50% over control animals, but both 1 and 5 g/kg of CERA-Q reduced the MDA increase to 10-20% over control values.

Histological analysis of brain tissue stained for tyrosine hydroxylase activity was used to determine loss of dopaminergic neurons and brain damage. CERA-Q treatment increased tyrosine hydroxylase positive cell numbers significantly compared to hydroxydopamine alone group. Improvements were about halfway back to normal, indicating protection of brain tissue and prevention of neuronal cell death by 1 and 5 g/kg oral CERA-Q.

Additionally, CERA-Q partly restored the decrease in non-activity time induced by imipramine in an animal model of depression – forced swim test.

Animal findings are important for several reasons. First, it showed that CERA-Q effectively improved memory impairment induced by muscarinic receptor antagonism. Second, peptides in CERA-Q were responsible for improvement in brain functions. Third, peptides had to cross the blood-brain barrier and improve cholinergic function in the brain. Fourth, individual peptides were somewhat more functional than CERA-Q itself, but not significantly so, suggesting a range of efficacy for different

peptides. Fifth, the three tested peptides were alanine-glycine rich to the same degree as CERA-Q itself (~75%). Finally, these effects were seen at a human equivalent dose of about 100 mg, which is less than the clinically studied does of 200 and 400 mg that also showed memory improvements. This provides strong evidence for a hydrophobic, small beta sheet peptide structure as key for mechanism of action.

CERA-Q In Vitro Effects on Brain Health

Beta Amyloid Interactions

Cultured human neuroblastoma SKN-SH cells and measured apoptotic changes, viability and Reactive Oxygen Species (ROS) generation after 24 hours of exposure to 20 µM beta amyloid 1-42 peptide (Chae et al., 2004; Kim et al., 2005). Histological examination revealed membrane blebbing, cell shrinkage, increased apoptotic nuclei and 40% neuronal cell death from beta amyloid. Addition of 10 µM CERA-Q two hours before beta amyloid addition normalized cell appearance and significantly prevented cell death (85% of control).

ROS measured three hours after beta amyloid addition found increased fluorescence that was significantly reduced by 10 µM CERA-Q and by 1 mM N-acetyl-L-cysteine (NAC). ROS generation (fluorescence) was 100% with beta amyloid, 20% for controls, 35% with CERA-Q + beta amyloid and 28% for NAC + beta amyloid. CERA-Q alone added to neuronal cell cultures did not exhibit deleterious effects. Thus, in vitro evidence illustrates neuroprotective effects and prevention of neuronal cell death from CERA-Q against damage from beta amyloid when both are present with neuronal cells.

Similarly, CERA-Q reduced beta amyloid apoptotic cascade events in vitro (Chae et al., 2004). SKN-SH neuroblastoma cells were treated with 10 µM CERA-Q for two hours then beta amyloid was added and after six hours, caspase-3 activity was measured. Control exhibited about 5% of beta amyloid activity (set to 100%). CERA-Q activity was 28%, while the pan-caspase inhibitor drug zVAD-fmk showed 20% activity. CERA-Q significantly reduced caspase activity (a prerequisite for apoptosis) similarly to a known inhibitor compound.

Choi and colleagues studied the effect of two µl of 50 mcg/ml CERA-Q in milk (CERA-Q milk) to SKN-SH neuroblastoma cells immediately with and after seven days with and without 10 µM beta amyloid (Choi et al., 2008). Beta amyloid caused a 50% decrease in cell survival and typical cell damage after 24 hours, similar to reports by Chae et al., 2004 and Kim et al., 2005. CERA-Q milk did not show adverse effects in SKN-SH cell culture. Addition of CERA-Q reduced cell damage and prevented cell death significantly, similar to earlier reports. This study intended to show that CERA-Q in milk still possessed neuroprotective properties as a prelude to human clinical testing.

Further research into anti-apoptotic mechanisms of CERA-Q against beta amyloid effects on neuroblastoma cells was conducted by DY Lee and colleagues (Lee et al., 2007). Using SKN-SH cells as before, additional steps in the apoptotic pathways were induced by the usual 10 µM beta amyloid exposure with and without CERA-Q at 10 femtomolar concentration (fM). Time-course of cell death was followed for 48 hours. CERA-Q did not affect cell viability, but when added with beta amyloid, cell viability was improved almost to normal control levels over 48 hours. Accordingly, caspase-3 activity was significantly decreased to the same degree as a caspase inhibitor drug (Ac-DEVD-CHO). Both CERA-Q and inhibitor drug returned capase-3 activity after 12 hours to 40% of beta amyloid activity (control was 20% of beta amyloid control set to 100%). Histological evidence of cell apoptosis (nuclear condensation and fragmentation) was significantly decreased by two hours of 10 fM CERA-Q after 24 hours of 10 µM beta amyloid. Intracellular calcium levels were significantly improved part-way to control levels by 10 fM CERA-Q and 100mM NAC. ROS generation was similarly affected by CERA-Q and NAC. Mitochondrial membrane potential was normalized at 6-12 hours by CERA-Q after beta amyloid treatment.

The results combined show that CERA-Q inhibited beta amyloid effects on initiation of caspase-mediated apoptosis and cell death. Thus, small amounts of

intact CERA-Q peptides inhibit beta amyloid effects on neuronal cells when present at the same time. For this mechanism to operate in vivo, CERA-Q peptides in active forms would have to cross the blood-brain barrier and reach beta amyloid peptides to interact. Whether this situation is extant in vivo remains to be directly determined, but animal feeding and i.p. studies suggest this mechanism is occurring.

Neuronal Cell Viability Models

Ferrous sulfate ($FeSO_4$) at 200 µM added to SKN-SH neuroblastoma cell cultures induced apoptosis via ROS production, and cell viability fell to 60% of control values (Lee et al., 2004c). Pretreatment with 10 µM CERA-Q for 24 hours restored cell viability to 90% of control culture levels in presence of $FeSO_4$. Histological observation showed anatomy of neuronal cells and apoptosis levels returned to normal when CERA-Q was added to $FeSO_4$, matching viability measures.

ROS generation in SKN-SH neuroblastoma cell cultures was increased 3.8 times by 200 µM $FeSO_4$. Addition of CERA-Q significantly decreased ROS generation to 1.2 times control values (not different from control).

CERA-Q exhibited inhibition of neuronal cell damage from two distinct methods of free radical production: 1) beta amyloid and 2) ferrous sulfate. This indicates that CERA-Q may operate on cellular defense mechanisms rather than direct effect on noxious agents, although an exact mechanism remains to be determined.

Scopolamine added to SKN-SH neuroblastoma cells also decreases cell viability to 50% of control cultures at 10 µM concentration (Kim et al., 2004). Typical neuronal cell signs of membrane blebbing, nuclear condensation and apoptosis were seen histologically after scopolamine. Adding 10 µM CERA-Q two hours before scopolamine led to increased cell viability – 90% of control, a significant difference from scopolamine-only values ($P<0.05$).

DH Kim and coworkers added 10 fM CERA-Q (as CERA-Q milk) to SKN-HS neuroblastoma cell cultures that were treated with 250 µM 3-hydroxykynurenine, a known endogenous stress agent for brain tissue (Kim et al., 2009a). CERA-Q prevented neuronal stress assessed by phase-contrast microscopy and normalized cell morphology.

Yeo and coworkers fractionated CERA-Q peptides into low (<500 daltons), medium (500-1000 daltons) and high (1000-2000 daltons) molecular weight ranges and tested each fraction for protection of PC12 neuronal cell cultures (Yeo et al., 2008). Cell viability after hydrogen peroxide treatment was 50% of control cultures. Low molecular weight range CERA-Q peptides performed better than medium or high fractions (73.6, 67.3 and 56.9% of control respectively). CERA-Q peptides were at 50 µg/ml concentrations. Low and medium ranges were significantly different from peroxide only group values.

Medium and high molecular weight ranges of CERA-Q peptides contained less glycine, alanine and serine, and more cysteine, phenylalanine and tyrosine than the low molecular weight fraction. This finding supports hydrophobicity of alanine-glycine rich peptides as a key determinant of CERA-Q function.

Nutrient Combinations with CERA-Q for Brain Health

CERA-Q silk fibroin peptide hydrolysate operates differently from other nutrients linked to brain health and mental performance. So far, nothing else has the same physical structures, nor evidence for a beta sheet mechanism of action (to be discussed below). Only milk has been published as an addition to CERA-Q for mental performance effects (Kim et al., 2009a) and cell culture effects (Choi et al., 2008). No diminution of CERA-Q effects was found – instead a relatively low dose of 10 mg showed similar effects to other human studies using 200-400 mg daily. Thus, CERA-Q exerts its effects when ingested with protein and energy calories (fat and carbohydrates). This brings up some interesting possibilities for

combinations with other nutrients to maintain healthy brain function. Almost any nutrient with beneficial effects for brain or mental functions can be added to CERA-Q with potential for additive or even synergistic effects. Only one of many conceivable combinations will be briefly presented.

CERA-Q & Caffeine

Caffeine is one of the most-studied phytonutrients for its effects on the human brain. Simultaneous doses of caffeine (200-400 mg) and CERA-Q (200-400 mg) conceivably could improve mental functions of alertness, motor control hand-eye coordination, focus, cognition, memory and perhaps mood and vigor.

CERA-Q improves brain circulation, which can deliver more glucose that caffeine spares (during exercise) and more fatty acids that caffeine releases – a potentially synergistic brain energetic action.

CERA-Q lowers elevated blood pressure in animal models, and in naïve individuals, caffeine may increase blood pressure. Although the interaction is unknown, there is a potential for CERA-Q to ameliorate cardiovascular effects of caffeine.

Even if an interference between caffeine and CERA-Q manifests, their different pharmacokinetic profiles would prevent interactions. Caffeine is absorbed and metabolized quickly after a single dose. CERA-Q exerts circulatory effects in the brain in a similar time period, which should accentuate each nutrients' effects. Mental performance effects of CERA-Q take longer to develop, within three weeks.

These actions of each nutrient suggest that formulations for skill sports, long-term endurance exercise and students studying for important classes or tests would be sensible. Of course, the value of CERA-Q combinations would need to be tested in order to assess safety and utility.

CERA-Q Mechanisms of Action

The following proposed mechanism for brain benefits from CERA-Q is derived from combined synthesis of basic protein structure biochemistry, cell biology, molecular biology and results from in vitro, animal and human studies.

CERA-Q = Quintessential Beta Sheet Peptides

Mechanism of action is simple, universal and revolves around interactions with other proteins from the distinctive alanine-glycine-rich primary structure (amino acid sequences) of the CERA-Q peptides. These sequences determine a quintessential beta sheet (ribbon-like) secondary protein structure, common to almost every protein. Beta sheet structures were identified in the 1930s (Astbury 1933) and defined as such in the early 1950s (Pauling & Corey, 1951).

To form a relatively stable, dimensionally flat ribbon structure, conserved and exact amino acid sequences are built into proteins so that amino acid chains can fold onto themselves or attach to other proteins – exact amino acid sequences "stick" to each other by electrostatic attraction. Other amino acids in key locations can make the stickiness stronger or looser or sheets large or smaller. Ribbons can be folded, twisted, turned, and modified by different amino acids in precise locations on the primary sequence into many different shape and size possibilities for protein chains. Sheets can "morph" into other basic secondary and tertiary structures such as fibrils and helices, offering a continuum of protein functionality possibilities.

Interactions of beta sheet sequences with other areas of a protein and other proteins are normal and common, both within a single protein and also between/among different or same proteins. Beta sheet interactions are a method to move, maneuver, position, attach/detach, and activate/inactivate proteins on a subcellular scale, allowing proteins to fulfill their intended functions.

While any amino acids can be in beta sheet sequences, some are better than others for

maintaining functional structures. CERA-Q peptides are different from other beta sheet sequences in other proteins. Sequences of CERA-Q alanine-glycine-rich peptides (2-35 amino acid residues) are the smallest functional units of beta sheet protein structure (Kang et al., 2013; see http://kinemage.biochem.duke.edu/teaching/anatax/html/anatax.2b.html for detailed review of protein structures). CERA-Q silk fibroin peptides are molecularly smaller than other beta sheet peptide sequences because of the small side chain size of alanine and glycine, the two smallest amino acids. Thus, CERA-Q peptides possess different properties, such as stronger hydrogen bonding, less steric hindrance, more penetration of polypeptide (protein) chains, and stronger hydrophobicity than other beta sheet peptides.

This is the key to understanding how CERA-Q works. A large amount of published scientific literature has accumulated on beta sheet peptides in general. Long story short, CERA-Q contains peptides that interact with beta sheet and other sequences in intact proteins by electrostatic attractions. Importantly, almost all membrane receptor proteins and membrane signaling receptors have beta sheet components integral to their activity and function, including receptor transport and turnover.

CERA-Q Peptide Actions – "Molecular Velcro®"
By virtue of their small size and structure, CERA-Q peptides are attracted to and/or bind to beta sheet portions of other proteins via electrostatic attractions between amino acids on each. In biological systems, the net effect is more or less mobility and reactivity of the beta sheet regions ("stickiness"). Receptor activity and turnover enhancement is one example of affectation. Enzyme activation, inhibition or control is another obvious corollary. So is prevention or slowing of aggregation of beta sheet structures into fibrils and tangles (ala amyloid plaque formation) or between different proteins (ala G protein receptor complexes assembly and disassembly).

Thus, CERA-Q can act like Velcro® to enhance receptor function, or it can act like a crowbar to prevent fibril formation of beta-amyloid protein by sticking to the beta sheet portion.

Anti-Beta-Amyloid Effects of CERA-Q & Beta Sheet Peptides
CERA-Q has at least one documented mechanism of action: prevention and/or disruption of amyloid plaque formation leading to amelioration of adverse brain effects, even after oral administration of typical dosages in animals (Chae et al., 2004).

A primary concern of brain function is normal loss of capabilities during aging or trauma (Selkoe 1994). Research has focused on Amyloid Precursor Protein (APP), a normal neuron membrane protein which is converted into smaller pieces to help protect and nurture neurons from diverse insults (Cardenas-Aguayo et al., 2014; Dawkins & Small 2014; Pearson & Peers 2006; Selkoe 1994; Zheng & Koo 2011). APP processing into smaller pieces is complicated and linked to synaptic activity. In normal amounts (picomolar), APP peptides/proteins, including beta-amyloid, protect brain cells by binding potentially toxic metals and reducing oxidative stress, which allows for normal memory formation and blood-brain-barrier integrity.

However, when confronted with a certain degree of injurious insults, APP processing shifts to formation of large amounts of beta-amyloid, released outside of brain cells (Cardenas-Aguayo et al., 2014; Dawkins & Small 2014; Pearson & Peers 2006; Selkoe 1994; Zheng & Koo 2011). This is where beta-amyloid can become neurotoxic. Beta-amyloid peptide is predominantly beta sheet sequences and structure (Antzutkin et al., 2000; Antzutkin et al., 2002; Balbach et al., 2002; Cerf et al., 2009; Chimon et al., 2007; Fasman et al., 1995; Halverson et al., 1991; LeVine 1999; Lovas et al., 2013; Petkova et al., 2004; Pike et al., 1995; Serag et al., 2002; Sikorski et al., 2003; Terzi et al., 1994; Zagorski & Barrow 1992). Under particular circumstances, the beta sheet sequences interact with each other and start folding beta-amyloid into fibrils, leading to a cascade of more aggregation into thicker fibrils, and eventually to amyloid plaque (Cardenas-Aguayo et al., 2014; Dawkins & Small 2014; Pearson & Peers 2006; Selkoe 1994; Zheng & Koo 2011). This process is not reversible, and is mediated by beta sheet

molecular chemistry. Beta sheet conversion to fibril formation is a key step in plaque formation.

Prions affecting brain health also become toxic when going through a beta sheet structure similar to beta amyloid peptides (Pan et al., 1993).

Interestingly, addition of alanine-glycine-rich CERA-Q (Chae et al., 2004; Kim et al., 2005; Lee et al., 2004b;) or other beta sheet peptides to beta-amyloid in vitro or fed or injected i.p. to animals prevented fibril and plaque and formation (Adessi & Soto 2002; Chacon et al., 2004; Findeis 2002; Lynn & Meredith 2000; Naito & Kawamura 2007; Permanne et al., 2002; Soto et al., 1998; Talaga 2001; Viet et al., 2011). Small peptides from 5-11 amino acids long (the same range as found in CERA-Q) are called beta-sheet-breakers. Synthesized versions are under investigation as drug treatments for Alzheimer's Disease. Beta-sheet-breaker peptides bind to the beta sheet areas of beta-amyloid, blocking ability to start the fibril/plaque formation cascade – an example of the "Velcro®" capability of beta sheet peptides. CERA-Q represents a "natural" series of beta-sheet-breaker peptides that help balance formation of toxic beta-amyloid fibrils, thus maintaining healthy brain function.

Importantly, CERA-Q has been shown to possess beta sheet peptide structures (Yao et al., 2004). Thus, CERA-Q can be considered as a beta-sheet-breaker peptide mixture.

A beta sheet mechanism for CERA-Q interactions with beta amyloid is evidenced by both in vitro and animal data from oral administration showing beneficial outcomes of brain biochemistry and organ function, as illustrated in CERA-Q Animal Studies (Chae et al., 2004; Kang et al., 2013; Kim et al., 2004; Kim et al., 2005; Kim et al., 2011; Lee et al., 2004; Lee et al, 2005; Yeo et al., 2004) and In Vitro Studies (Chae et al., 2004; Choi et al., 2008; Kim et al., 2004; Kim et al., 2005; Kim et al., 2009; Lee et al., 2007) sections earlier in this document.

Non-brain Effects of CERA-Q Peptides

A corollary of beta sheet interaction mechanism for CERA-Q is that other biological effects can be predicted. There is evidence that CERA-Q exhibits additional cell signaling effects in other tissues throughout the body. In fact, biological effects in other tissues are presented in the scientific literature for CERA-Q, silk fibroin peptides and purified or synthetic versions of characteristic, single peptides in CERA-Q.

Preclinical beneficial effects for circulatory function (blood pressure control), blood sugar regulation (insulin, glucose and diabetes), bone health and anticancer effects have been published from various laboratories around the world. Combined, the results support a beta-sheet mechanism of action for CERA-Q based on alanine-glycine-rich peptides.

Blood Pressure Effects of CERA-Q Peptides

Preclinical beneficial effects for blood pressure control via Angiotensin I converting enzyme (ACE) inhibition have been reported in both in vitro and animal model studies for CERA-Q and some of its component peptides (Igarashi et al., 2006; Ni et al., 2001; Vercruysse et al., 2005; Wang et al., 2008; Zhou et al., 2010). For example, two small peptides isolated from CERA-Q reduced systolic blood pressure as well as Captopril® in Spontaneously Hypertensive rats (Igarashi et al., 2006).

Glucose and Insulin Resistance Effects of CERA-Q Peptides

Hyun and coworkers exposed 3T3-L1 adipocytes to excess insulin to reduce insulin-stimulated glucose uptake (Hyun et al., 2004). Exposure of 3T3-L1 cells to fibroin peptides blocked the response to insulin and increased sensitivity of control cells to insulin, especially at physiological concentrations. Fibroin peptides did not alter post-receptor signaling cascade (important for safety reasons). Glucose metabolism and glycogen turnover was increased independent of insulin. GLUT1 was upregulated and GLUT4 translocation enhanced. These results show a receptor control and enhanced efficiency action for fibroin peptides equivalent to CERA-Q.

Kim and coworkers treated 3T3-L1 cells with synthesized peptides found in CERA-Q silk fibroin hydrolysates (Kim et al., 2009b). Hexapeptides exhibited the strongest activity on GLUT-4

translocation without affecting GLUT-4 synthesis. Thus, CERA-Q peptides are insulin-sensitizing agents that block insulin resistance.

Chon and coworkers administered silk fibroin hydrolysate to 3T3-L1 cell cultures and found decreased adipocyte differentiation, increased glycerol release, upregulation of fatty acid oxidation enzymes and downregulation of adipogenic enzymes PPARγ and C/EBPα (Chon et al., 2010). These results suggest that CERA-Q peptides might have applications for treatment of obesity.

Similarly, silk fibroin peptides and three synthetic silk fibroin peptides were added to 3T3-L1 preadipocyte cell cultures (Huang et al., 2010). Differentiation was affected and proteomic analysis found 15 protein expressions changed by silk fibroin peptides.

Lee and coworkers also demonstrated increased glucose uptake via upregulation of GLUT-4 and reduced leptin expression in 3T3-L1 cells (Lee et al., 2011).

Genetically diabetic mice (C457/Ksj/db/db strain) were fed silk fibroin protein hydrolysate at 100 or 200 mg/kg for four weeks (Jung et al., 2010). Plasma glucose, glycated hemoglobin , total cholesterol, LDL cholesterol, atherogenic index were significantly decreased. Plasma insulin levels were increased, and pancreatic Islet cells exhibited increased insulin staining. These results suggest that CERA-Q peptides improve reduced insulin output by pancreatic beta cells, providing amelioration of diabetic profiles.

Genetically diabetic mice (C457/Ksj/db/db strain) were fed CERA-Q in their drinking water (Do et al., 2012). At various times afterwards, mice were examined for pancreatic Islets of Langerhans Beta cells. The CERA-Q group showed less loss of Beta cells, increased Beta cell growth factors and less Beta cell death signals (apoptosis markers).

Park and coworkers found that 20% silk fibroin hydrolysate in drinking water fed to C457/Ksj/db/db mice showed improvement in plasma levels of glucose and lipids, similar to other studies (Park et al., 2013).

Interestingly, an earlier study did not find significant lowering of glucose in C457/Ksj/db/db mice, but did find improvements in glucose and body weight in streptozotocin diabetic rats fed silk fibroin peptides (Park et al., 2002). Feeding peptides in the diet at 1-3% was different from mice fed in single doses per day or in drinking water, and may have accounted for success in later studies in C457/Ksj/db/db mice.

Thus, CERA-Q exerts beneficial effects on glucose and insulin metabolism, via receptor turnover and cell signaling effects. Of interest is improved insulin production of pancreatic Islet beta cells. The results were reproducible and extrapolate to practical human equivalent doses. No adverse effects on diabetic animals was found. Thus, CERA-Q has potential practical importance for diabetic conditions, and potential for more efficient utilization of glucose in normal settings such as exercise, pending human study findings.

Bone Health Effects of CERA-Q Peptides

Culture medium from M3T3-E1 osteoblasts treated with 10-100 mcg of silk fibroin peptides for six days was added to RAW-264.7 cell cultures for three days along with RANKL to induce osteoclastic differentiation (Yeo et al., 2008). Fibroin peptides inhibited osteoclastic differentiation and expression of interleukin -1β.

Silk fibroin hydrolysate showed direct inhibition of RAW 264.7 osteoclast differentiation in vitro studies via inhibition of nuclear factor κB ligand-activated receptor function (Chon et al.,2012). Spontaneous apoptotic signaling cascades were induced by the hydrolysate. These effects were similar to those of Yeo et al., 2008, and suggest that CERA-Q peptides can decrease osteoclast formation, a potentially beneficial action for long-term bone health.

Low molecular weight peptides resembling biodegraded products of silk implants used in bone tissue engineering resembled CERA-Q (Kim et al., 2010a). Addition of silk low molecular weight peptides (1-100 mcg/ml) to cultures of osteoblastic MG-63 cells increased expression of genes involved in new bone formation. Thus, silk scaffolding may

support new bone formation by release of peptides also found in CERA-Q.

Ovariectomized rats were given alendronate (10 mg/kg), 100 or 300 mg/kg silk fibroin peptides (analogous to CERA-Q) orally for 12 weeks (Kweon et al., 2015). Both groups of silk fibroin peptides showed equivalent results to alendronate for increased bone mineral density and 3-dimensional bone imaging. Markers of bone status were also similar to alendronate, except for alkaline phosphatase and osteocalcin, which resembled sham control levels. Overall, silk fibroin peptides effectively prevented trabecular bone loss after hormone loss in female mice at human equivalent doses starting at ~800 mg daily. Intervention outcomes of increased bone formation by CERA-Q peptides supported the results of previous in vitro studies.

Other Potential CERA-Q Mechanisms

Another potential mechanism is simply mass action of amino acid supply to cells in need. However, in general for amino acid supplies, this mechanism needs relatively large amounts of oral peptides (gram amounts) to influence protein synthesis. It is certain that the ultimate fate of CERA-Q and all peptides is breakdown to component amino acids. This mechanism may be operative but is overshadowed by the interactions discussed above.

Since alanine and glycine are major components of CERA-Q peptides, it is possible that additional supply of these two amino acids together to cells can increase overall energy metabolism and ATP production, which would allow a global improvement in cell, tissue, organ and organism performance. Again, at the doses used, it is not likely that metabolic effects of alanine and glycine are predominant for CERA-Q mental effects.

Similarly, CERA-Q peptides could provide needed amino acids that improve production (or prevent breakdown) of key regulatory proteins and/or metabolites or pathways in cells and tissues. Again, it is unlikely that the clinically studied doses are sufficient to improve this potential mechanism, although it could be additive.

CERA-Q Mechanism Summary

In summary, the most likely, but not the only, mechanism to account for CERA-Q results is that CERA-Q operates on a basic electrostatic charge mechanism to assist control of widespread and important receptor functions and amyloid protein aggregation. Other mechanisms related to subcellular amino acid delivery or interactions with cell signaling pathways are possible. These potential mechanisms can account for clinically observed results from human studies on enhancement of brain functions, as well as preclinical findings on blood pressure, bone and insulin metabolism.

CERA-Q & BRAIN EFFECTS – SUMMARY

In conclusion, the unusual beta sheet amino acid sequences of CERA-Q silkworm cocoon fibroin protein hydrolysate peptides operate on a universal, reversible and versatile molecular level of electrostatic attractions that interact beneficially with important signaling and receptor proteins that help maintain normal cellular and tissue functions that affect brain activity. In addition to supportive evidence from preclinical investigations, high-quality human intervention outcome RCTs with normal, healthy persons uniformly demonstrate significant improvements from baseline and from placebo for attention, cognition, concentration, focus, intelligence, memory, spatial organization and brain circulation at doses of 200 and 400 mg daily at three weeks and longer. No adverse events have been reported for CERA-Q, safety studies have not found potential for adverse effects and CERA-Q falls under USFDA GRAS status. Thus, CERA-Q is a premier nutrient for maintenance and/or improvement of normal, healthy brain functions in all ages.

Luke R. Bucci, PhD CCN CNS
Vice President, Product Innovation

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