

Zila Nutraceuticals Ester-C[®] Research Summary

Introduction

At Zila Nutraceuticals, we are constantly looking for new and innovative ways to find out how our product benefits the human body. Traditional methods of research include both *in vitro* and *in vivo* studies. The advantages of *in vitro* studies, which are done with cultured cells as opposed to live animals or humans, are many. Since these studies can be completed quickly and economically, much information can be gained in a relatively short period of time. For example, a certain nutraceutical product could be shown to inhibit the growth of cultured cancer cells. The disadvantage of these studies is that there is no way to show the same effect would be seen in a human being, since the issues of absorption, distribution, metabolism, and excretion have been bypassed. It is also possible to expose cultured cells to a much higher concentration of a product than would be feasible through oral ingestion. *In vivo* studies, which include both animal and human clinical trials, are much more definitive but are more time consuming and expensive. Full-blown human clinical trials can take many years and millions of dollars to complete. Successful *in vitro* studies would generally precede human clinical trials.

One of the new technologies becoming available to research is called “gene expression technology.” The goal is to see how products affect people at the genetic level. That is, to see which genes have been stimulated (“up-regulated”) or inhibited (“down-regulated”) by the product. Two techniques are usually employed to study the genetic effects of products. Before describing these techniques, it may be useful to very briefly talk about genes. Strands of deoxyribonucleic acid, or DNA, make up our genes. The gene is DNA made of unique sequences of individual nucleic acids. This unique sequence “codes” for unique RNA (ribonucleic acid) that “codes” for specific proteins. The proteins perform many functions in the body, and the study of protein expression and how proteins affect physiological functions is called proteomics.

As mentioned earlier, there are two main techniques used to study gene expression. While it is beyond the scope of this article to explain how they work, I will briefly describe them. The first method utilized DNA microarrays. I call this more of a “shotgun” approach to gene studies because 5000-10000 or more genes can be studied in a single experiment. The second method utilized PCR (polymerase chain reaction). If the researcher knows the genes believed to be modulated by the product, then a more targeted approach utilizing PCR technology may be used. Unlike DNA microarrays, real time quantitative PCR allows for gene expression to be quantified so that the degree of up or down regulation is known relative to a control. Cells are exposed to either the test product or a control. After sufficient time the RNA is extracted from the cells. If the targeted gene was up regulated, there will be more copies of the

corresponding RNA extracted. Then, through sophisticated methods, the relative number of RNA copies between the treatment and control can be measured (through reverse transcription to cDNA and amplification by PCR). We are currently using these advanced techniques to study the effect of Ester-C on genes that modulate the immune system. The new techniques, combined with traditional *in vitro* and human clinical trials, continue to reveal new ways Ester-C promotes good health.

Absorption and Retention of Ester-C

Vitamin C (L-ascorbic acid) is critical to over 300 bodily functions. Rapid cellular uptake and delayed renal excretion of ascorbic acid is conducive to providing optimum cellular concentration for biochemical activity. Several studies have been performed to test the hypothesis that the unique structure of Ester-C renders it more readily absorbed and less rapidly excreted than the acid or salt form of the vitamin.

In a study at the University of Mississippi School of Pharmacy¹, Ester-C and L-ascorbic acid were administered to two groups of rats. Blood was sampled at 20, 40, 80, 160 and 240 minutes, and analyzed for ascorbic acid. As urine appeared in collection cups, it was tested qualitatively for the presence of ascorbic acid.

The plasma concentration of ascorbic acid was higher at 20, 40 and 80 minutes in the rats treated with Ester-C than in the rats given L-ascorbic acid. Ascorbic acid was detected twice as early in the urine of animals administered with L-ascorbic acid than those treated with Ester-C. Less rapid excretion, concurrent with higher plasma values of ascorbic acid, is consistent with the hypothesis that Ester-C provides better cellular concentration for biochemical activity..

The results of this investigation suggest that Ester-C may be advantageous in increasing circulating ascorbic acid concentration and promoting rapid tissue saturation.

A human bioavailability study involved three groups of men all on a diet that excluded citrus, green leafy vegetables and vitamin supplementation for one week prior to the start.² At the beginning of the study, blood was drawn from each participant to determine serum and white blood cell ascorbate levels.

The three groups were given daily doses of either:

¹ Bush, Marilyn J. and Verlangieri, Anthony J. (1987). An Acute Study on the Relative Gastro-Intestinal Absorption of a Novel Form of Calcium Ascorbate. Research Communication in Chemical Pathology and Pharmacology 57 (1).

² Wright, Jonathan V, M.D. and Suen, Raymond M. (1990). Comparative Studies of "Ester C" Versus L-Ascorbic Acid. International Clinical Nutrition Review 10 (1).

Ester-C, 4000 mg (3000 mg of calcium ascorbate)
L-ascorbic acid, 3000 mg
Citric acid, 3000 mg

After ingestion, blood specimens were taken at 0, 4, 8 and 24 hours to determine serum and white blood cell ascorbate levels. Twenty-four hour urine specimen collections were taken and analyzed for ascorbate and oxalate.

The three groups were switched after a 2-6 day washout period, and blood and urine specimen collections were made and analyzed at exactly the same intervals as before.

The second Ester-C and L-ascorbic acid groups continued taking these supplements at the same levels for 7-10 days, at the end of which fasting blood specimens and 24-hour urine collections were taken.

Results of the Ester-C groups compared to the L-ascorbic acid groups showed:

- significantly higher serum levels than L-ascorbic acid at 4, 8 and 24 hours after ingestion, as well as after 7-10 days of continuous ingestion.
- significantly higher white blood cell levels at 8 and 24 hours after ingestion and after the 7-10 days of continuous ingestion.
- substantially less oxalate excretion in the first 24 hours after ingestion, as well as significantly less oxalate excretion after 7-10 days of continuous ingestion.
- substantially less urinary ascorbate loss in both the first 24 hours of ingestion and after the 7-10 days of continuous ingestion.

The Metabolites in Ester-C

The metabolism of ascorbic acid involves its oxidation to dehydroascorbic acid, followed by the formation of the aldonic acids L-lyxonic acid, L-xylonic acid, and L-threonic acid. Several researchers have studied whether it is the metabolites in Ester-C that are the mechanism by which Ester-C is readily absorbed and retained by the body. One such study investigated the effect of calcium L-threonate, the major calcium aldonate found in Ester-C.³

This study was done using human cutaneous T- lymphoma cell line. In the experiments, the cells were serum starved to deplete levels of serum proteins such as insulin, which has been shown to stimulate cellular ascorbic acid uptake. The cells were then suspended in 1.0 ml of calcium L-threonate of varying concentrations and L-[1-¹⁴C] ascorbic acid was added to a final ascorbic acid concentration of approximately 1.25 mg%.

³ Fay, Michael J. and Verlangieri, Anthony J. (1991). Stimulatory Action of Calcium L-Threonate on Ascorbic Acid Uptake by a Human T-Lymphoma Cell Line. Life Sciences 49 1377-1381.

The results in Table 1 show that calcium L-threonate increased cellular ascorbic acid accumulation, with the highest dose increasing uptake 177% over control values. A similar study with the L-tartaric acid, an aldonic acid not derived from ascorbic acid metabolism, did not show increased uptake of ascorbic acid (unpublished data from the same authors).

Table 1

Ca Threonate mg%	DPM/10⁶ Cells	% Increase
0 (control)	1,662 ± 83	0
100	2,302 ± 199	39
500	2,915 ± 130	75
750	4,177 ± 567	151
1,000	4,610 ± 597	177

A follow up study further tested the effect of L-threonic acid and L-lyxonic acid on ascorbic acid uptake using 3T3 mouse fibroblasts and human T lymphoma cells.⁴ This study also tested the effect of potassium chloride, L-tartaric acid, ouabain, and 2,4-dinitrophenol on ascorbic acid uptake, with the following results:

- Calcium L-threonate augmented ascorbic acid uptake by the fibroblasts in a dose-related manner, with the 23 and 30.5 mM doses being significant from the 0mM control group.
- The potassium salt of L-lyxonic acid at 30.5 mM produced a 23% increase in ascorbic uptake in fibroblasts, but increased ascorbic acid 198% above the control group in T-lymphoma cells. (This effect was not due to potassium ion, as the uptake ratio was significantly greater than that of potassium chloride.)
- There was a significant decrease in ascorbic acid uptake when tartaric acid was present.
- Ouabain and 2,4-dinitrophenol significantly decreased ascorbic acid uptake by the T-lymphoma cells and prevented calcium L-threonate from augmenting ascorbic acid uptake; these substances did not significantly affect uptake or appear to inhibit the ability of calcium L-threonate to increase uptake in the fibroblasts.

These results indicate some specificity on the part of threonate and lyxonate in terms of ascorbic acid uptake and suggest that they may have a modulatory effect on ascorbic acid transport.

⁴ Fay, Michael J. et al. (1994). Effect of Aldonic Acids on the Uptake of Ascorbic Acid by 3T3 Mouse Fibroblasts and Human T Lymphoma Cells. Gen. Pharmac. 25 (7) 1465 –1469.

To further test the theory that the metabolites in Ester-C facilitate ascorbate activity in the body, tests were done with non-ascorbate synthesizing rats.⁵ Two groups of rats, initially fed a diet with only trace amounts of ascorbic acid, were supplemented with either ascorbic acid or Ester-C in distilled drinking water. After 24 days, the following results were observed:

- The Ester-C group had a significantly greater increase in body weight – 125% compared to 46% increase in the ascorbic acid group.
- There was a high correlation between ascorbate activity equivalents and body weight gain when the ascorbate equivalents were administered as Ester-C.
- The ascorbic acid group had consistently higher scorbutic scores than the Ester-C group.
- Two rats in the ascorbic acid group were so morbid by day 24, that death was expected from scurvy within a day or two. There was no morbidity in the Ester-C group.

Cellular Accumulation of Ester-C

It has been suggested that the plasma level of vitamin C only reflects the vitamin in transit, on the way to be delivered to critical target cells. Thus, studies of cellular accumulation and biochemical functions are important in interpreting advantages offered by different forms of vitamin C. A recent, not yet published human study was completed at the University of California, Los Angeles, to determine white blood cell levels of vitamin C after oral administration of ascorbic acid, placebo, and two preparations of Ester-C.⁶

Three groups of five subjects were randomized in a double-blind cross-over clinical study. After an initial washout period of low vitamin C diet, each was given one of the three vitamin C preparations or placebo, in random sequence, separate by washout periods. Blood samples were collected at 0, 1, 2, 3, 4, 8 and 24 hours after each administration, yielding the following results:

- The two Ester-C preparations led to longer retention of vitamin C in the white cells, compared to ascorbic acid; the longer retention was evident 24 hours after administration.
- The lowest levels of white cell vitamin C were observed among smokers, showing more rapid drops in levels compared to non-smokers, after administration of ascorbic acid. However, smokers retained significantly higher levels of white cell vitamin C after administration of the Ester-C preparations.

⁵ Verlangieri, Anthony J. et al. (1991). Comparison of the Anti-Scorbutic Activity of L-Ascorbic Acid and Ester-E in the Non-Ascorbate Synthesizing Osteogenic Disorder Shionogi (ODS) Rat. Life Sciences 48 2275-2281.

⁶ Bernal, Samuel et al. Vitamin C Uptake in White Blood Cells in Vivo (unpublished).

Ester-C and Immunity

A study using high-precision molecular analysis of gene expression, developed by Source Precision Medicine, was utilized to explore changes in the expression of select immunomodulatory genes in response to Ester C.⁷

Using a single human donor, Ester-C was evaluated in whole blood treated with and without exogenous stimuli (untreated whole blood) *in-vitro*. Ester-C was observed to stimulate key immunomodulatory genes in a concentration dependent fashion (6.25, 12.5, 25, 50, 100 and 200 ug/ml) in untreated whole blood. For example, Ester C exhibited a concentration dependent activation (3 to 10 fold) for IL1RN, a gene known to play an important role in regulating the immune response.

Furthermore, Ester-C was found to augment the response of whole blood to two key inflammatory and immunological stimuli - bacterial endotoxin (LPS, lipopolysaccharide) and cytokine IL1B. Specifically, CXCL2, IL10, IL1A and PLA2 were modulated in a concentration dependent fashion. At 200 ug/mL (equivalent to a 1g dose in man), Ester-C was observed to block the LPS induction of IL10 by >90% and augment the CXCL2, IL1A and PLA2 response >6-fold. (LPS is a potent activator of innate immune responses, and in human blood it stimulates a broad-based inflammatory/immune response. IL1B is known to be involved in a variety of inflammatory and immunological responses, including the response to infection.)

Finally, a preliminary study in man (n=1) showed an *in-vivo* whole blood response from a single oral dose of Ester-C two and four hours post-dose, relative to pre-dose. Together, these data help define the *in-vitro* and *ex-vivo* dynamic state of whole blood in man in response to Ester-C. Potential correlations between these dynamic states warrant further study.

⁷ Ester-C Form of Vitamin C Modulates the *In-Vitro* Response of Human Whole Blood to Two Key Inflammatory and Immunological Stimuli