

# Cell-based Antioxidant Protection

## A novel method of the evaluation of natural products

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### Abstract

The objective of this research was to examine the value of a novel **cell-based antioxidant protection in erythrocytes (CAP-e)\*** assay for the evaluation of whether antioxidants in natural products are capable of penetrating and protecting live cells from oxidative damage.

The assay uses basic chemical principles, similar to those used with the ORAC test, to measure specific effects of products on a highly simplistic cell type, the erythrocyte (red blood cell). The advantage of utilizing the erythrocyte for the assay is that this cell type does not perform cellular signaling, undergo apoptosis, or produce reactive oxygen species, whereas all other cell types used in cell-based assays can.

We have tested natural products with known ORAC values, and found that certain products such as the freeze-dried Acai berry, with an extremely high ORAC of 1,027  $\mu\text{mol}$  Trolox equivalents per gram, also provided good protection of cells from oxidative damage. We have also identified a bovine colostrum whey-based extract, Immunel®, with a known ORAC value of 18  $\mu\text{mol}$  Trolox equivalents per gram, which was able to protect cells better than would be expected based on the product's ORAC value alone.

Furthermore, we used a version of the CAP-e method to test serum samples from a clinical pilot study, testing antioxidant uptake after consumption of placebo versus MonaVie Active™, a juice rich in Acai. The CAP-e assay was able to detect an increase in serum antioxidants, and the data correlated with changes in lipid peroxidation as measured by the TBARS test.

We are currently performing a single laboratory validation for the assay, using a select panel of natural products of plant, animal, and microbial origins.

*\* Patent pending.*

### Introduction

Free radicals are generated as a result of normal metabolism, as well as by stress caused by disease and environmental pollution. As a part of the growing interest in nutritional strategies for anti-ageing and disease prevention, antioxidants in foods and nutritional supplements have become a major area of focus. Antioxidant testing has become a cornerstone of the natural products industry. One of the most frequently utilized methods is the Oxygen Radical Absorbance Capacity (ORAC) assay.

Neither the ORAC assay nor other chemical-based antioxidant tests provide information regarding the bioavailability of antioxidants to living cells, organs, or organisms. The ideal study for proving that antioxidants in a product are absorbed by a living being (whether animal or human) is a clinical bioavailability trial. Such studies are highly complex, costly, and must take into account the effects of the digestive process on the chemical nature of compounds. Another method of clinically proving antioxidant uptake is to test whether consumption of a product actually contributes to antioxidant protection in a whole living being, whether animal or human.

Once a product is characterized by the ORAC test panel, further information is needed to examine various

aspects of bioavailability. In other words, do the antioxidants have any meaningful impact for those who consume them? This includes addressing the following questions:

- Is the antioxidant absorbed upon consumption?
- Local effects in gut tissue?
- Entry into blood circulation?
- Entry into living cells?
- Ability to protect cells from oxidative damage?

The current investigative options include:

- a) A full clinical trial focused on assessment of inflammation/pathology;
- b) A clinical trial to evaluate the antioxidant capacity and/or inflammatory markers in serum;
- c) Animal testing;
- d) Cell-based testing.

Seeking faster and economical assays as an addition to existing tests such as the ORAC causes a turn towards cell-based laboratory testing. The pharmaceutical industry has set standards for the first three experimental models; however, no standardization exists regarding cell-based testing. Numerous cell types are used by laboratories and many cell-based assays are too complex to provide simple answers regarding antioxidant uptake into live cells. NIS Labs has developed an inexpensive assay [5,6], which addresses one simple, yet very important question: **Can a specific antioxidant enter and protect a living cell?**

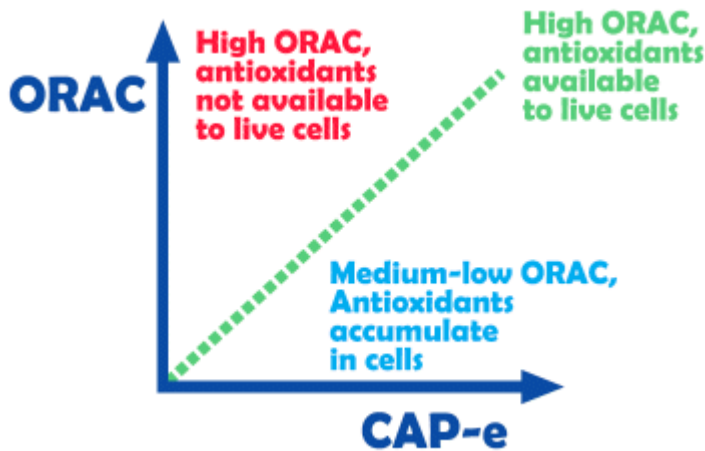


Figure 1. Diagram showing several possible situations pertaining to antioxidant capacity of natural products, when comparing data obtained by ORAC versus CAP-e: a) A product may have a high ORAC value but be unable to enter and protect cells from oxidative damage; b) a product may perform well in both assays; and c) a product may show better protection of cells than what would be expected based on the ORAC value alone.

## Method

The CAP-e assay is based on a methodology similar to the ORAC test, but is performed in cells of a very simple composition. The assay makes use of erythrocytes (predominantly human, but dog and horse cells have also been used). In contrast to other cell-based models, the CAP-e assay utilizes erythrocytes (red blood cell) because they do not contribute to oxidative damage like other cells commonly used, and the assay specifically measures those antioxidants capable of crossing the plasma membrane into the intracellular space.

In this assay, the cells are exposed to test products in physiological saline. The cells are allowed time to absorb compounds from the test product. Any compounds not absorbed by the cells during that period are removed by centrifugation and subsequent washing. The cells are exposed to a precursor dye that becomes fluorescent when exposed to oxidative damage. Subsequently, the cells are subjected to an oxidative challenge such as H<sub>2</sub>O<sub>2</sub> or AAPH. The fluorescence intensity reflects the amount of oxidative damage. As a

positive control, cells are exposed to oxidative challenge without any antioxidant protection, and serve as a measure of maximal oxidative damage. Any reduction of oxidative damage to the cells pre-treated with the test product reflects antioxidant protection. The assay is qualitative in principle, but does allow for some semi-quantitative comparisons to standards such as Gallic acid, Trolox, and Ascorbic acid.

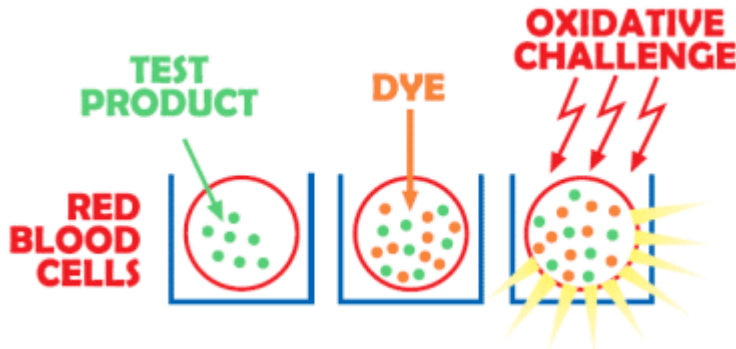


Figure 2. Methods principle for the CAP-e assay. Erythrocytes (red blood cells) are exposed to a natural product to allow antioxidants to enter into the cells. Unabsorbed antioxidants are removed, and the cells are loaded with a precursor dye. Upon an oxidative challenge, the precursor dye emits fluorescent light in proportion to the amount of oxidative damage. A reduction of fluorescence is proportional to antioxidant protection.

## Comparison to other cell-based assays

The CAP-e constitutes a cell-based model for antioxidant testing that neither has the complexity of the PMN/monocyte assay, nor the risk of misinterpretation possible with tumor cell-line-based assays.

### **Inhibition of production of reactive oxygen species (ROS) by polymorphonuclear leukocytes (PMN) or primary monocytes/macrophages.**

When it has been determined by the CAP-e assay to what extent and at which concentration anti-oxidant constituents in a natural product are able to cross the plasma membrane of red blood cells and offer protection from oxidative challenge, it is prudent to proceed with testing in the ROS PMN assay. The purpose is to examine the effect the test product has on cells types that in contrast to RBCs play a role in inflammatory processes.

PMN cells are complex and capable of reacting in several ways upon exposure to natural products as follows:

1. Passive absorption of antioxidants into the cells, leading to neutralization of ROS within the cells;
2. Active signaling by compounds in the natural product (for example glucans) leading to increased ROS production;
3. Active signaling by compounds in the natural product leading to a reduced inflammatory response by the cell and therefore a reduced production of ROS.

This assay comprises the measurement of these three possible cellular responses to treatment with the test article and provides a summarization of the simultaneous effects.

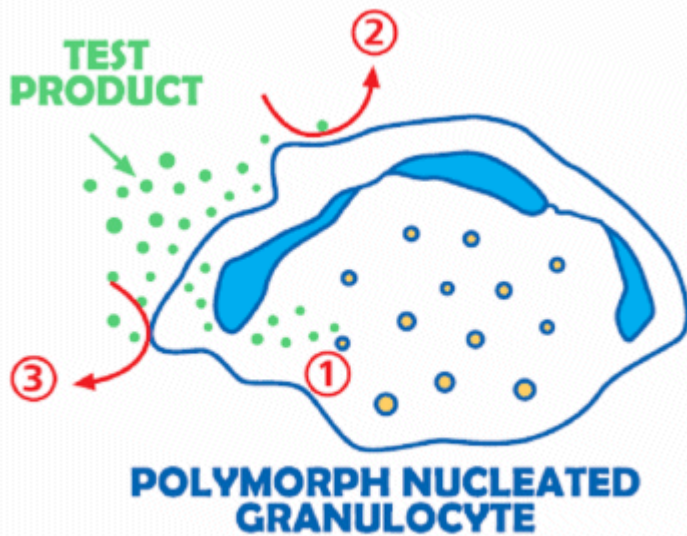


Figure 3. Methods principle for the ROS PMN assay. The essential methodology is similar to the CAP-e, except that the cell type used is the inflammatory PMN cell. This cell type can respond to natural products in the following three ways: 1) Antioxidants enter the intracellular space and neutralizes ROS; 2) Compounds within the natural product may bind to receptors on the cell surface and mediate a signal without entering the cell, leaving the cell less able to secrete ROS; 3) Compounds within the product may bind to other types of cell surface receptors, and trigger signaling towards a more pro-inflammatory behavior, leading to increased ROS formation.

### ROS production in cell lines

The choice of immortalized cell lines for the purpose of antioxidant testing is not a straightforward approach. Immortalized cell lines—which include many commonly used tumor cell lines—offer some consistency for assays that test natural products. Although it may be argued that this eliminates the problem associated with minor variations in blood samples drawn from healthy donors for cell-based testing, many tumor cell lines proliferate in a highly deregulated manner. This can lead to a proportion of the cells in the culture being the result of asymmetrical cell division, which can result in apoptosis (programmed cell death) and thus introduce inherent variability in the cell population.

ROS are produced by tumor cells as a result of programmed cell death. A reduction in ROS formation may actually reflect a reduction cell death in these cell lines in the presence of a test product. Of course, increased survival or proliferation of a tumor cell line is not an ideal marketing claim—especially because it may have little clinical relevance.

Despite their poor suitability for testing of antioxidant capacity, tumor cell lines lend themselves to other types of natural products testing. The use of tumor cell lines for evaluation of tumor-suppressive effects is more straightforward, and can include the following assays, either separately or in combination: Inhibition of cell proliferation; effects on mitochondrial functioning; and induction of programmed cell death (apoptosis).



Figure 4. ROS production in tumor cell lines take place as part of programmed cell death (apoptosis). Reduction of ROS formation in tumor cell lines may reflect increased survival of the tumor cells in the presence of a natural product.

## Results

### Protection from peroxy versus hydroxyl radicals in the CAP-e assay.

As part of our validation of the CAP-e assay, we have performed repeated testing of the polyphenolic-rich Amazonian palm berry known commonly as Acai (*Euterpe oleracea*). Our source of freeze-dried (FD) Acai (K2A, Provost Utah) has a well characterized antioxidant capacity. It contains a high level of phenolic compounds, a very high hydrophilic ORAC (997  $\mu\text{mol}$  Trolox equivalents per gram), a high lipophilic ORAC (30  $\mu\text{mol}$  Trolox equivalents per gram), and a high level of superoxide anion scavenging capacity (1,614 mg Gallic Acid equivalents per gram) [3].

A novel bovine colostrum-based extract, Immunel™ (Sterling Technology Inc., Brookings SD), was also tested by the CAP-e assay. Immunel™ has a hydrophilic ORAC value of 18  $\mu\text{mol}$  Trolox equivalents per gram.

Both FD Acai and Immunel™ have previously been tested in the ROS PMN assay, but with opposite effects. The FD Acai triggered a highly significant reduction on ROS formation by PMN cells [3], whereas Immunel™ triggered a mild but statistically significant increase in ROS formation by PMN cells ( $P < 0.03$ ) [7]. Therefore, it was of interest to compare such different immunomodulatory products with the CAP-e assay.

The FD Acai provided statistically significant antioxidant protection to live cells, as demonstrated in the CAP-e assay, with  $P < 5 \times 10^{-10}$  for the dose responsible for 50% inhibition of maximal oxidative damage ( $\text{IC}_{50}$ ). When the oxidative challenge phase of the CAP-e assay was performed by adding the peroxy radical generator, 2,2'-Azo-bis-(2-amidinopropane)-dihydrochloride (AAPH), Acai provided much greater protection than when the oxidative challenge was induced by the hydroxyl radical generator hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The  $\text{IC}_{50}$ (AAPH) for FD Acai was 3.3 mg/mL.

Immunel™ was also highly capable of protecting cells from oxidative damage demonstrated by the CAP-e assay ( $P < 0.003$  at the  $\text{IC}_{50}$  dose). Immunel™ provided better protection against peroxy than hydroxyl radicals, as demonstrated by using AAPH and  $\text{H}_2\text{O}_2$  in parallel to trigger oxidative stress. The  $\text{IC}_{50}$ (AAPH) for Immunel™ was 65 mg/mL. The result highlights that a complex natural product such as Immunel™ can provide support for the innate immune response by supporting ROS formation, while simultaneously providing protection from oxidative damage.

Additional research is in progress to compare a broader range of natural products with known ORAC values to results from the CAP-e assay.

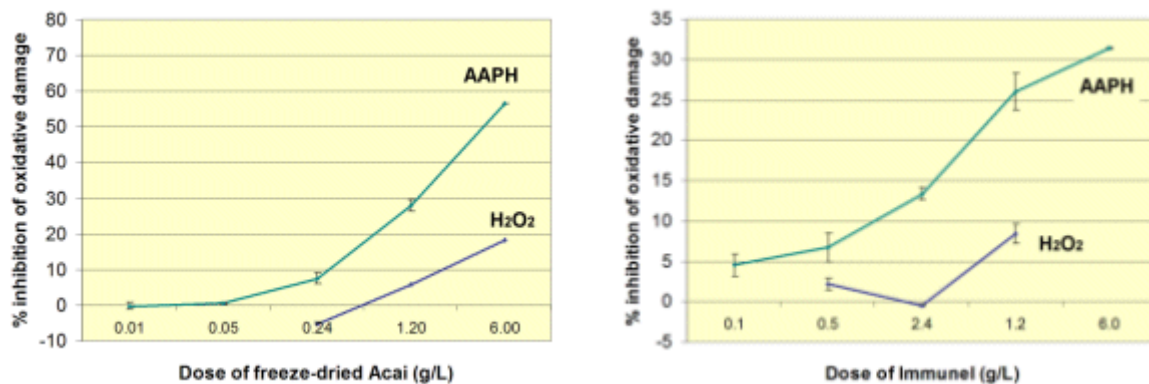


Figure 5. CAP-e results from testing of antioxidant capacity of two test products: Freeze-dried Acai and Immunel®. The oxidative challenge was performed either by the peroxy radical producer AAPH (green lines) or the hydroxyl radical generator  $\text{H}_2\text{O}_2$  (blue lines).

## Clinical application of the CAP-e test

We have recently applied the CAP-e assay methodology to serum samples collected from a randomized, double-blinded, placebo-controlled, cross-over pilot study of 12 healthy adult subjects [4]. The purpose of this study was to investigate the *in vitro* and *in vivo* antioxidant capacity of MonaVie Active™, a juice primarily consisting of Acai. Blood samples were obtained at baseline as well as one and two hours following consumption of either 4 oz. (120 mL) of the juice or a placebo—which consisted of encapsulated purple-colored potato flakes.

The CAP-e test demonstrated an increase in the serum antioxidant level, which at two hours post-consumption showed a greater than 65% correlation with another method of measuring oxidative stress, namely TBARS—a measure of MDA (malondialdehyde), which is proportional to the level of serum lipid peroxidation. MonaVie Active™ consumption resulted in an increase in serum antioxidants at one hour ( $P < 0.03$ ) and at two hours ( $P < 0.015$ ), as well as a reduction in serum lipid peroxidation (TBARS) within two hours ( $P < 0.01$ ).

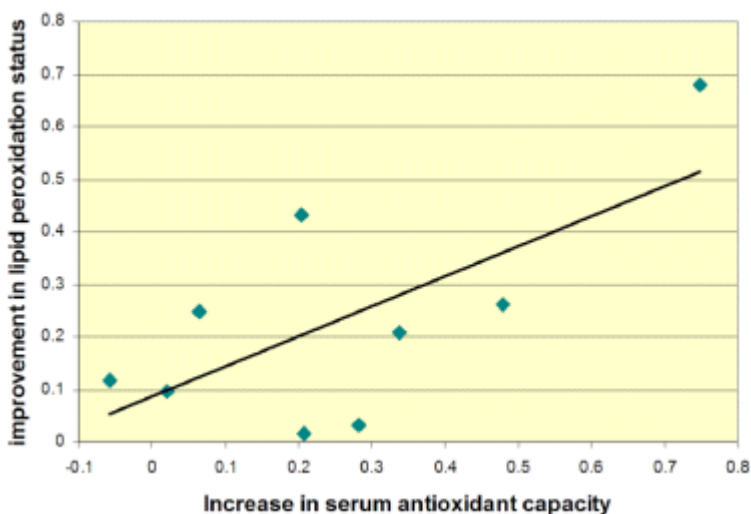


Figure 6. Correlation between increase in serum antioxidant capacity as measured by the CAP-e assay (X axis), and the reduction in lipid peroxidation measured by the TBARS assay (Y axis), at 2 hours after consumption of either 4 oz MonaVie Active® juice or a placebo. Data were obtained from a cross-over study. Individual data were normalized to the baseline level prior to consumption, and subsequently the differences in changes after consumption of Placebo versus MonaVie were calculated. There was a 67% correlation between antioxidant uptake versus reduction in lipid peroxidation.

## References

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