Eniva Research Group

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Research Summary:

Investigation of cellular membrane protection by a proprietary phytonutrient health nutraceutical (VIBE 2.0 Cardiac & Life) against lipid peroxidation assault on human cells via hydrogen peroxide and peroxyl radicals.

1. Introduction & Summary:

It is well known that oxidizing agents, including reactive oxygen species (ROS), can promote the initiation and propagation of free radical damage to lipids, key components of cellular membranes. The oxidation of lipids within a cellular membrane is known as lipid peroxidation. Lipid peroxidation increases with age and is known to play a significant role in cardiovascular, dermatologic and degenerative issues. Should this damage be within blood-vessel-lining endothelial cells or low density lipoproteins (LDL), this can contribute to arterial plaque development. Due to these facts, the identification of substances which can offer defensive attributes against cellular membrane assault and damage are desirable to identify and characterize.

This investigation undertook the task of evaluating a nutraceutical product (Eniva VIBE 2.0 Cardiac & Life™) containing verified RDA essential nutrients, proprietary blends of fruit, vegetable, aloe vera gel and a green tea epigallocatechingallate (EGCG) catechin complex for its ability to impact free radical peroxide levels and attenuate free radical assault within the lipid-rich intra-membrane environment of human keratinocytes. This was done through the use of the lipid peroxidation assay utilizing DCFH-DA for fluorescence (assay performed by MB Research, PA). Hydrogen peroxide was selected as the exogenous oxidizing initiator of lipid damage. Cytotoxicity was also assessed. A variety of concentrations of the Eniva health supplement were tested (0.01%, 0.1% and 1%) in the assays. Results demonstrated a statistically and biologically significant reduction in cellular peroxide levels at all concentrations tested when compared to control. The greatest reduction was 66% at the 1% test material (V/V) concentration.

It is clear from the data that the Eniva VIBE 2.0 Cardiac & Life nutraceutical demonstrates a biologically significant ability to reduce the level of peroxides within the cellular membrane environment associated with exogenous assault as compared to control. This result suggests a potential role in protecting cellular lipid-rich membranes from attack by exogenous reactive oxygen species. Due to these results, the VIBE 2.0 Cardiac & Life health supplement could play a role in the promotion of healthy aging as well as in the specific areas of cardiovascular and dermatologic health.

2. Experimental Design:

Method Synopsis:

Human epidermal keratinocytes (from adult donor, HEKa) received from Cascade Biologics were grown to 70-80% confluence. Cells were removed from the plate (approx. 70% confluent) with Trypsin-EDTA, washed and resuspended (to approximately 4 x 10^5 cells/ml) in Dulbecco's Phosphate Buffered Saline (DPBS, 25 C). The peroxide-specific dye DCFH-DA (10 µM, final concentration) was then added to the cell suspension and the cells were incubated in a water bath (35 C) for 10 minutes.. Cells were washed again and resuspended in DPBS to the previous volume. Aliquots (0.25 ml) of the dye-loaded cells were transferred to 12 x 75 mm polypropylene tubes containing 0.25 ml of either DPBS alone (vehicle control), Trolox (positive control), or various (increasing) concentrations of the test article (treatments were conducted in triplicate). Two sets of vehicle controls were conducted, at the start and at the end of the study. The sample tubes were then returned to the water bath for an additional 5 minutes. Sets of samples were treated with or without hydrogen peroxide (H2O2, final concentration 60 µM) for 15 minutes. The test tube rack containing the samples was then rapidly placed into ice water. Propidium iodide was added to label non-viable cells. The peroxide-specific fluorescence in control samples and in samples treated with test article (+ H2O2 treatment) was then determined using a flow cytometer.

Test Material: Eniva VIBE 2.0 Cardiac & Life Nutraceutical. Verified ingredient listing on file.

Sample preparation:

The test article was shaken to ensure a homogeneous suspension. 80 µl of the test article was added to 3.92 ml of Dulbecco's Phosphate Buffered Saline (DPBS) to yield a 2% solution. 400 µl of the 2% solution were added to 3.6 ml of DPBS to yield a 0.2% solution. 400 µl of the 0.2% solution were added to 3.6 ml of DPBS to yield a 0.02% solution. Final concentrations of 1%, 0.1% and 0.01% were achieved by adding the cells to each solution.

Cell Culture Methods:

Human epidermal keratinocytes from adult donor (HEKa, Lot #5C0622) were received from Cascade Biologics (Portland, Oregon) on 09/20/06. When needed, the cells were trypsinized, counted and then plated and grown to 70-80% confluence in T-75 flasks in growth medium (EpiLife, Cascade Biologics), to obtain a sufficient quantity of cells for the peroxide assay experiment.

Labeling and Treatment:

For peroxide determination, cells were trypsinized, washed and resuspended (approximately 4-6 x 10 cells/ml) in Dulbecco's Phosphate Buffered Saline (DPBS, 25 C). The peroxide-specific dye DCFH-DA was then added to the cell suspension and the sample was incubated in a 35 C water bath for 10 min. Cells were washed again and resuspended in DPBS to the previous volume. Aliquots of the cells were transferred to 12 x 75 mm polypropylene tubes containing either DPBS alone (vehicle control), Trolox (positive control), or various concentrations of the test article. The cells were then returned to the water bath for an additional 5 minutes. The Sample set was then treated with hydrogen peroxide for 15 minutes. The test tube rack containing the samples was

then rapidly placed into ice water. Propidium iodide was added to label non-viable cells. The peroxide-specific fluorescence in control samples and samples treated with test article was determined by flow cytometry using a Becton-Dickinson FACScan flow cytometer. Using this method, the effect of a test article on exogenous peroxide levels can be measured.

Control Groups:

The vitamin E analog, Trolox (Aldrich, Cat. #238813), which is known to exhibit antioxidant properties, was used as a positive control in these studies. This agent was included in samples at a final concentration of 300 µM. Two sets of vehicle controls were conducted, at the start and at the end of the study. The difference between the vehicle controls was used in the calculation to correct for fluorescence drift across the samples. All control groups were conducted +/- H 2O 2 treatment.

Cellular Peroxide Levels:

The peroxide-specific (green) fluorescence of the cells was measured using a Becton-Dickinson FACScan flow cytometer. Debris was excluded from the analysis using an electronic gate that was created on a dual-parameter light scattergram (i.e., linear forward angle vs. linear 90-degree light ("side") scatter).

Data Presentation:

The peroxide-specific fluorescence of the cells was determined (in triplicate) using flow cytometry and the Mean Fluorescence Intensity (MFI) was calculated (+/- SD) for each data set. The data were also presented as percent of control. A treatment group was considered to be "biologically significantly different from control" if the peroxide levels were less than 75% or greater than 125% when compared to vehicle control (25% change). The effect of the test article on the influence of extracellular peroxides on cellular peroxide levels was expressed as the percent of the control +60 µM H2O2. In some cases, a control group was used for comparison with several test articles.

Viability Analysis:

Approximately 15-30 minutes prior to flow cytometric analysis, the samples were treated with the DNA- specific (red fluorescent) dye, propidium iodide. This dye is excluded from living cells. Using flow cytometry, an electronic gate is set to include whole cells and the percentage of non-viable cells that were labeled with this dye was then determined. It must be noted that this method can provide a good estimation of the percentage of cells that may be abruptly killed by the acute treatments. However, it is possible that the test article may disrupt the association between the dye and the DNA of dead cells (which is dependent on hydrogen binding) to give spuriously low results, underestimating the percentage of dead cells in the gated portion of the sample.

3. Results:

1. Cytotoxicity:

- The Vibe 2.0 Cardiac and Life nutraceutical did not demonstrate cytotoxicity to the human cells tested. The 1% concentration data is likely under represented as there was increased flow debris during experimentation.

Table 1: Effect of Eniva VIBE 2.0 Cardiac & Life, Lot/Batch #218302, on cell viability expressed as percent of non-viable gated cells

Concentration	Treatment	Series 1	Series 2	Series 3	Std Dvtn	Mean
Negative Control	No H202	1.45	2.25	2.53	0.56	2.08
Negative Control	+60 umol H202	4.06	3.74	2.00	1.11	3.27
VIBE 0.01%	+60 umol H202	4.43	2.25	4.04	1.16	3.57
VIBE 0.1%	+60 umol H202	2.94	2.95	3.59	0.37	3.16
VIBE 1%	+60 umol H202	0.00	0.01	0.08	0.04	0.03
Positive Cntrl: 300 uM Trolox	+60 umol H202	5.59	3.72	5.01	0.94.	4.77

2. Antioxidant activity, cellular membrane assault and lipid peroxidation:.

- The Vibe 2.0 Cardiac and Life nutraceutical demonstrated antioxidant activity and had a statistically significant decrease in exogenous peroxide levels as demonstrated by a decrease in intra-membrane cellular peroxide values as compared to control. As this assay represents a picture of intra-membrane lipid assault due to the positioning of the DCFH molecule, it can be stated the test material demonstrated a defensive mechanism against lipid peroxidation.

Table 2: Effect of Eniva Vibe (2.0), Lot/Batch# 218302, on Cellular Peroxide Levels / Peroxide-Specific Fluorescence

Concentration	Treatment	Series 1	Series 2	Series 3	Std. Dvtn	Mean
Negative Control	No H202	46.25	47.85	46.75	0.82	46.95
Negative + H202	+60 umol H202	224.51	212.74	221.71	6.15	219.65
VIBE 0.01%	+60 umol H202	149.89	146.49	157.63	5.71	151.33
VIBE 0.1%	+60 umol H202	103.80	99.89	111.48	5.9	105.06
VIBE 1%	+60 umol H202	79.06	78.83	71.82	4.12	76.57
Positive Cntrl: 300 uM Trolox	+60 umol H202	68.22	64.21	63.82	2.44	65.41

Figure 2: Impact of VIBE Cardiac & Life formulation on peroxide radical generated fluorescence:

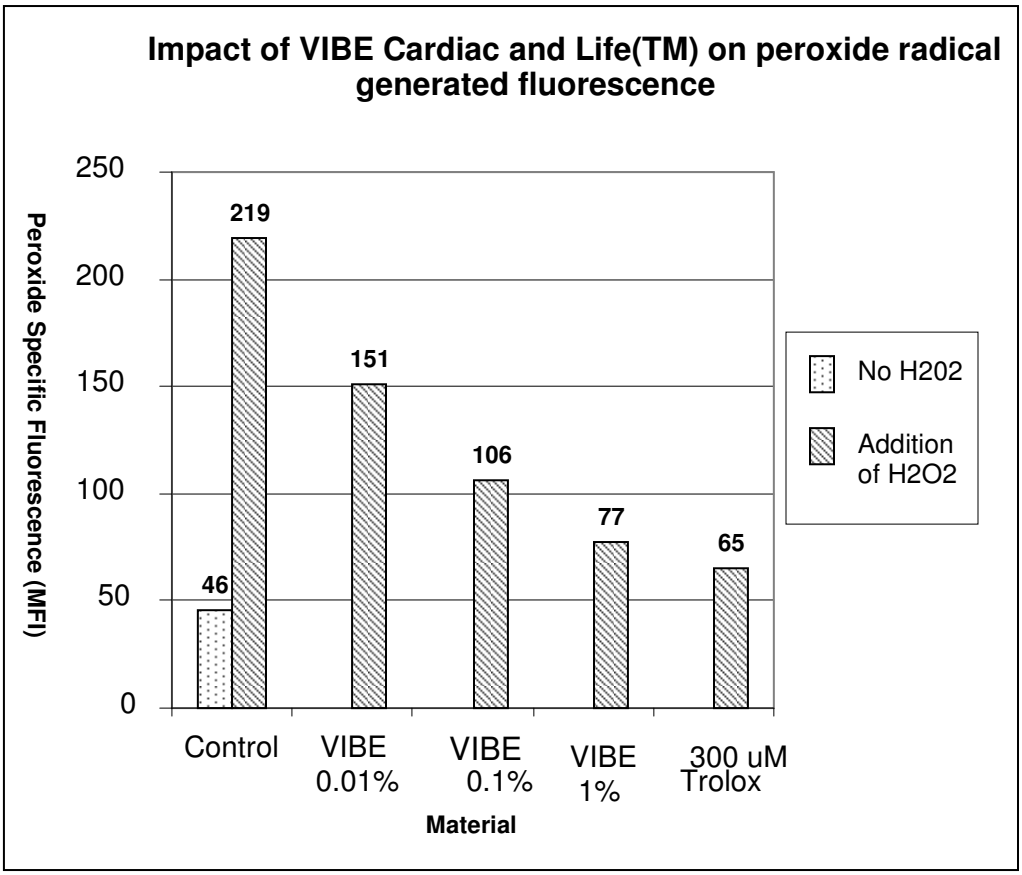
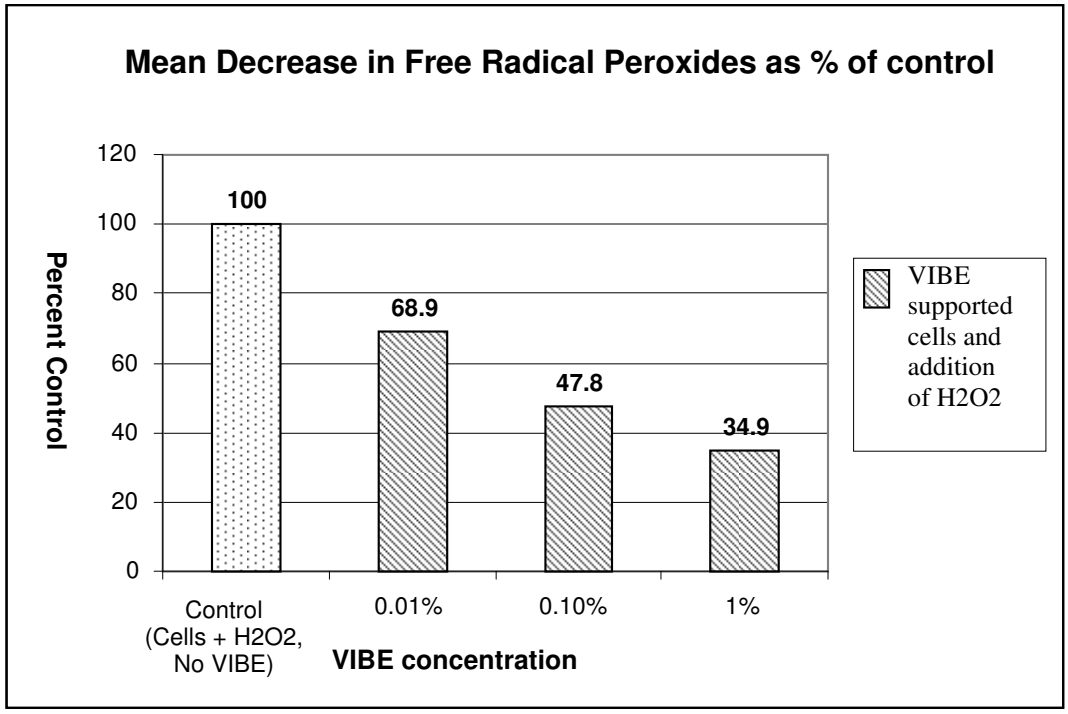


Table 3: Summary of peroxide levels as a percent of control, with biologic significance identified at 25% increase or decrease as compared to control

Test Article	Concentration	% control w/H202	> 25% Increase or Decrease in Peroxide Level
Vibe 2.0 Cardiac & Life	0.01%	68.9	DECREASE
	0.1%	47.8	DECREASE
	1%	34.9	DECREASE

Figure 3:



*** Biological significance noted at all VIBE concentrations tested.

4. Results: Eniva Vibe 2.0 Cardiac & Life

1. Cytotoxicity:

- The material tested did not demonstrate significant cytotoxicity to the cells tested. Cellular debris was elevated at the highest concentration tested. Levels of mean viability were greater than 95% at all concentrations tested and was not significantly different than control.

2. Antioxidant activity and cellular membrane protection from lipid peroxidation assault.

- The material tested demonstrated antioxidant activity against hydrogen peroxide and associated free radicals generated.
- The material tested demonstrated a statistically and biologically significant reduction in exogenously initiated peroxide levels.
- The material tested demonstrated a defensive mechanism against exogenous cellular membrane assault and associated lipid membrane peroxidation.

5. Discussion

1. Cytotoxicity

In an era with there is intense investigation into potential substances and compounds which have health promoting properties, it is important to evaluate these same substances for cytotoxicity. In this investigation, evaluation of the VIBE 2.0 Cardiac & Life nutraceutical found the substance to be non-toxic to human cells. While cellular debris was elevated at the highest concentration tested during flow cytometry and could potentially indicate a mild under representation of non-viable cells, the remaining results and running dynamics support the VIBE 2.0 Cardiac & Life material as non-toxic. Cell viability at all concentrations tested was greater than 95%.

2. Antioxidant activity and cellular membrane protection from lipid peroxidation assault.

Lipid specific cellular membrane damage is well-known to increase with aging and play key roles in cardiovascular, dermatolgogic and degenerative health conditions. Regarding cardiovascular impact, significant lipid peroxidation can contribute to arterial plaque formation and potential ischemia secondary to decreased blood flow. When this occurs in cardiac tissue, myocardial infarction may occur. The strong oxidizing agent hydrogen peroxide and other related reactive oxygen species are well-known to contribute to this type of cellular membrane damage.

The role of antioxidant substances has been heavily investigated in relation to their ability to help stabilize and protect cellular membranes. In this study, the antioxidant capacity of the VIBE 2.0 Cardiac & Life nutraceutical was investigated against hydrogen peroxide and associated reactive oxygen species, such as hydroxyl and peroxyl radicals. The results demonstrate the VIBE 2.0 Cardiac & Life nutraceutical possesses antioxidant capacity against hydrogen peroxide and its associated reactive oxygen species assault on lipid membranes. This suggests a role for this nutraceutical in the promotion and maintenance of healthy cellular membranes in the body. While multiple body systems can be included, especially cardiovascular and dermatologic, the product's demonstrated reduction in lipid peroxides also points to a potential role in cholesterol metabolism as relates to a potential protective mechanism against the oxidation of low density liporoteins (LDL). Additional investigation in this area is warranted.

The specific assay used 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). This substance has been widely used in research studies for its known ability to be oxidized by hydrogen peroxide and associated peroxyl radicals from the process of lipid peroxidation. The associated oxidation of 2,7-dichlorodihydrofluorescein to the fluorescent substance 2,7-dichlorofluorescein (DCF) can then be quantified as a measure of ROS levels/activity and provide insight into the occurrence of lipid peroxidation. It has been previously suggested that DCFH-DA would undergo subsequent acetylation by intracellular esterases and then the resulting DCFH would react with intracellular hydrogen peroxide or other oxidizing ROS at the *interface* of the lipid bilayer with the internal aqueous environment, providing florescence via the 2,7 dichlorofluorescein (DCF) compound.

However, recent research has put into serious question this proposed mechanism. Research-based NMR (nuclear magnetic resonance) chemical shift polarity correlation has determined that DCFH-DA and DCFH are located well within the lipid bilayer and certainly likely not at the interface of the lipid bilayer with the internal aqueous environment of the cell. Due to this important mechanistic component of the assay, this investigation specifically points to the impact of the reactive oxygen species directly within the lipid-bilayer level of the outer cellular membrane. While contributions from both the extracellular and intracellular environments influence this intra-membrane location, this intra-membrane location of the DCFH-DA molecule lends itself well to evaluating exogenous substances which can cross and potentially harm the lipid rich cellular membrane. This harm would occur via lipid peroxidation while the substance was on its way to the intracellular compartment, should it be able to fully cross. One such substance is hydrogen peroxide. Hydrogen peroxide is well known to cross cellular membranes and to also serve as an initiator of lipid peroxidation propagation via initiation of other radicals. With the use of exogenous oxidants like hydrogen peroxide in experimental designs, the assay more accurately represents the dynamics of intra-membrane exogenous lipid assault and associated response by intra-membrane components, whether they be inherent or traveled, rather than a representation of direct interaction with components within the intracellular compartment.

Relating this to cardiovascular issues, the lipid-rich cellular membrane of blood-vessel-lining endothelial cells, which represent the most outer exposure to exogenous oxidants, is a key locale in the early phase of the atherosclerotic cascade. In this study, there was a statistically significant decrease in the level of fluorescence and associated peroxide radical levels as compared to control in the cells supported with the VIBE 2.0 Cardiac and Life formulation, very likely representing a decrease in the ROS *within* the lipid rich intra-membrane environment. This decrease represents a protective characteristic of the VIBE nutraceutical against reactive oxygen species assault on the lipid membrane of the cells tested.

It is clear from data presented the VIBE 2.0 Cardiac and Life nutraceutical demonstrates a biologically significant ability to reduce the level of peroxides within the cellular membrane as compared to control. Due to the fact that lipid peroxidation and generalized oxidation of cellular membrane components is a key process in aging and many cardiovascular, dermatologic and degenerative conditions, the results would suggest that further investigation of the VIBE 2.0 Cardiac and Life nutraceutical as a substance which may offer defensive attributes against cellular membrane assault is warranted. .

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