

The Investigation of Antioxidant Properties of ALKAZONE[®] Electrolyte Antioxidant Mineral Supplement

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Abstract: Antioxidants are molecules that interfere the oxidation of other molecules. Oxidation is a chemical reaction that generates free radicals, leading to chain reactions that damage cells. For years, it has been claimed that alkaline water with antioxidants can increase the antioxidant capacity, and so protect the cells from oxidative stress. But, not many publications have been documented with proven-scientific methods. This study was performed as the first step in a series of laboratory and clinical evaluations for the effects of Alkazone[®] Electrolyte Antioxidant Mineral Supplement. The data here in this study demonstrated that the ALKAZONE[®] Electrolyte Antioxidant Mineral Supplement has antioxidant capacity, and also contributes a protective effect to living cells under oxidative stress. Further work is recommended for more elaborate cellular models, as well as in human clinical studies for anti-inflammatory activity and free radical stress.

Keywords: Antioxidants, Mineral Supplements, Alkazone Mineral Drops, Alkaline Drinking Water, Folin-Ciocalteu Assay, CAP-e Assay

1. Introduction

In ancient oriental medicine, some health-promoting protocols include an attempt to enhance the pH of the blood and urine via diet and drinking water. Alkaline drinking water has been used in folk medicine throughout history. Examples include Native American use of water from sulfur springs and alkaline lakes for medicinal and ritual purposes. The research for the effects of acidic and alkaline pH on mammalian cell function is an active area, and has shown that cells can sense acidity via receptors for protons (H⁺). Acidification of blood and tissue is associated with free radical stress on the body, inflammatory processes such as asthma, and support of cancer development and progression. Enzymes such as alkaline phosphatase, crucial for regulating cellular signaling processes, work optimally at alkaline pH. These are indicators that a local alkaline tissue environment may have favorable effects on healthy regulation of cellular functions.

It is noteworthy that pluripotent stem cells, important for regenerative processes, contain very high levels of alkaline phosphatase. Very little is currently known about how fully differentiated, nucleated mammalian cells function at alkaline pH. It has been shown that pH affects cellular membrane elastic properties, using a non-nucleated red blood cell model [1]. Recent publications have studied the role of pH in maintaining pluripotential capacity of human bone marrow stem cells. In one model system, pH above 7.54 fully inhibited osteogenic differentiation, and maintained the cells in an undifferentiated state [2]. In regenerative medicine, peripherally related observations include the demonstration that mesenchymal stem cells kept under reduced oxygen levels enhanced their long-term maintenance of the pluripotential state, but also reduced the ability to differentiate towards bone formation [3].

The study hypothesized that a moderately alkaline environment may promote antioxidant and anti-inflammatory protection in cells and tissue, and may potentially support healthy cellular signaling and re-programming in response to

external and endogenous signals. This project contributes an initial step towards generating a research-based portfolio to document the cellular and clinical effects of ALKAZONE® Electrolyte Antioxidant Mineral Supplement. Work performed A strategic sequence of research projects has been built, and this work represents the initial step in this sequence. Product handling The ALKAZONE® Electrolyte Antioxidant Mineral Supplement for this project: abbreviated to AMD (Alkazon mineral drops) are in suspension in an aqueous solution. In preparation for adding to laboratory assays, the AMD were diluted in distilled water according to the instructions for consumption: Three drops of AMD were added to 8 oz. water, and then serial dilutions prepared from there. A wide dose range was tested for cellular stress prior to adding the AMD solutions.

2. Experimental Methods

2.1. Tests Performed

The goal for this initial project was to generate a foundation of basic understanding of the actions of the product. The following work was performed as part of this project: Documented pH and ORP measurements in distilled water, physiological saline, phosphate-buffered saline (PBS), testing antioxidant capacity (Total Phenolics Assay), and testing cellular antioxidant protection (CAP-e Assay).

2.2. Methodological Details

2.2.1. PH Meter Readings

The ALKAZONE® Electrolyte Antioxidant Mineral Supplement was added to distilled water, physiological saline and phosphate-buffered saline (PBS). Using the directions from the package, 3 drops of ALKAZONE® was added to 8 ounces (250 mL) of each liquid. The pH meter (VWR Scientific Products) calibration was verified with three known standards before use (pH 4, 7, and 10) on each day of testing. Each liquid with 3 drops of ALKAZONE® was mixed thoroughly, measured, and recorded for pH.

2.2.2. ORP Meter Readings

The ALKAZONE® Electrolyte Antioxidant Mineral Supplement was added to distilled water, physiological saline and phosphate-buffered saline (PBS). Using the directions from the package, 3 drops of ALKAZONE® was added to 8 ounces (250 mL) of each liquid. The Oxidation Reduction Potential (ORP) meter (Smart Portable ORP Meter range ± 999 mV) was placed in each liquid after mixing thoroughly. The meter was allowed to stabilize and results were measured and recorded for Oxidation Reduction Potential. Interpretation of ORP results: a negative number means the solution is anti-oxidative; a positive number means it is oxidative.

2.2.3. Antioxidant Capacity Using the Folin-Ciocalteu Assay

The product was tested in the Folin-Ciocalteu assay (also known as the total phenolics assay). This assay makes use of

the Folin-Ciocalteu reagent to measure antioxidants. The assay is performed by adding the Folin-Ciocalteu's phenol reagent to serial dilutions of extract, thoroughly mixing, and incubating for 5 minutes. Sodium carbonate is added, starting a chemical reaction producing a color. The reaction is allowed to continue for 30 minutes at 37°C. Optical absorbance is measured at 765 nm in a colorimetric plate reader. Gallic acid is used as a reference standard, and the data reported in Gallic Acid Equivalents per gram product.

2.2.4. Cell-Based Antioxidant Protection Assay (CAP-e Assay)

The rationale [4] behind the method that we use is important: It allows assessment of anti-oxidant potential in a method that is comparable to the ORAC test, but only allows measurement of antioxidants that are able to cross the lipid bilayer cell membrane, enter the cells, and provide biologically meaningful antioxidant protection under conditions of oxidative stress. We developed the CAP-e bioassay specifically to work with natural products and ingredients [5]. The method has been used on multiple types of natural products and ingredients, published in the peer-reviewed scientific literature [6, 7, 8, 9, 10].

As a model cell type, the red blood cell (RBC) is used. This is an inert cell type, in contrast to other cell types such as PMN cells. This assay was particularly developed to be able to assess antioxidants from complex natural products in a cell-based system.

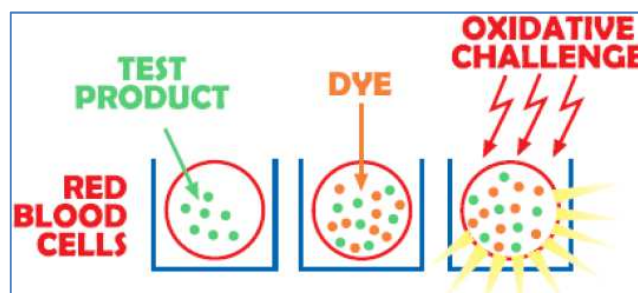


Figure 1. The illustrative diagram for presenting the procedures of CAP-e assay.

Freshly purified human RBC are washed repeatedly in physiological saline, and then exposed to the test products. During the incubation with a test product, any antioxidant compounds able to cross the cell membrane can enter the interior of the RBC. Then the RBC are washed to remove compounds that were not absorbed by the cells, and loaded with the DCF-DA dye, which turns fluorescent upon exposure to reactive oxygen species. Oxidation is triggered by addition of the peroxy free radical generator AAPH. The fluorescence intensity is evaluated. The low fluorescence intensity of untreated control cells serve as a baseline, and RBC treated with AAPH alone serve as a positive control for maximum oxidative damage.

If a reduced fluorescence intensity of RBC was exposed to a test product and subsequently exposed to AAPH, this indicates that the test product contains antioxidants available

to penetrate into the cells and protect these from oxidative damage.

3. Results and Discussions

3.1. PH and ORP Meter Readings

In preparation for product handling for the antioxidant assays for this project, it was necessary to test the consequences for diluting the test product in either distilled water, physiological 0.9% saline, or phosphate-buffered saline (PBS), both with respect to alkalinity and cellular tolerance.

The ALKAZONE® Electrolyte Antioxidant Mineral Supplement was added to distilled water, physiological saline and PBS. Using the directions from the package, 3 drops of

ALKAZONE® were added to 8 ounces (250 mL) of each liquid.

Below is the table of results for pH, oxygen reduction potential (ORP), and lysing (rupture) of red blood cells. Crenation of red blood cells was observed by microscopy. It is known that water will rupture red blood cells, but with the addition of ALKAZONE®, some red blood cells survived, although they all showed crenation. Crenation is the formation of abnormal notching around the edge of a red blood cell. The notched appearance of a red blood cell is due to its shrinkage after suspension in a hypertonic solution. The pH varied as did the ORP readings. The oxygen reduction potential (ORP) showed distilled water and physiological saline to be antioxidative by the negative numbers observed whereas phosphate buffered saline was oxidative.

Table 1. The pH, ORP and lysis of red blood cells in incubating solutions.

3 Drops ALKAZONE in 250 mL	pH	ORP	Lysis	Crenation
Distilled Water	10.2	-0.33	50%	All cells crenated
Physiologic Saline	9.6	-0.05	N/A	15% of cells crenated
Phosphate Buffered Saline	8.1	0.18	N/A	15% of cells crenated

This testing clearly showed that diluting the test product in phosphate-buffered saline altered the oxygen reduction potential (ORP), so it was no longer negative, and thus not likely to carry an antioxidant effect.

The use of distilled water was harsh on the red blood cells as expected, but since the cellular antioxidant protection assay makes use of 6 serial dilutions across a wide dose range, seeing cellular lysis and crenation at the highest dose was not surprising, and as can be seen on the data graphs below, the lower doses of test product in distilled water were tolerated by the cells in the assay. The decision was made to focus this testing on product handling using distilled water and physiological saline, and avoid using the phosphate-buffered saline for the subsequent testing.

3.2. Antioxidant Capacity in the Folin-Ciocalteu Assay

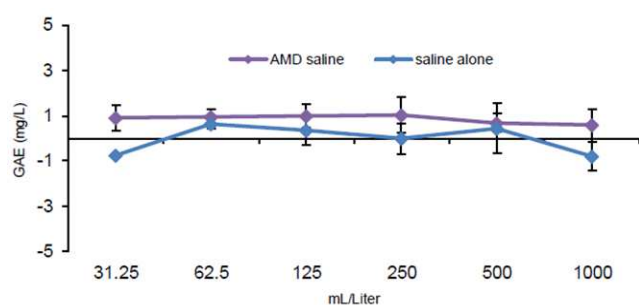


Figure 2. Antioxidant Capacity of Alkazone® mineral drops in saline.

Both Alkazone® mineral drops in saline and saline alone (control) showed some antioxidant capacity in this assay. At all concentrations Alkazone® mineral drops showed higher antioxidant capacity than saline alone.

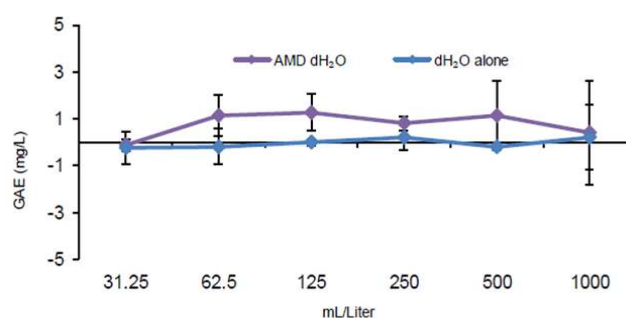


Figure 3. Antioxidant Capacity of Alkazone® mineral drops in distilled water.

Alkazone® mineral drops in distilled water showed some antioxidant capacity in this assay near to the unit 1 at GAE unit on average. At all concentrations Alkazone® mineral drops showed higher antioxidant capacity than distilled water alone, except at the highest and lowest concentration tested. The distilled water control stayed very near baseline throughout the doses tested.

3.3. Cellular Antioxidant Protection (CAP-e)

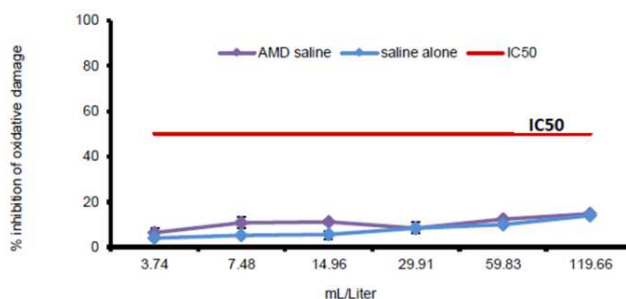


Figure 4. Cellular Antioxidant Protection with Alkazone® mineral drops in saline.

Both Alkazone[®] mineral drops in saline and saline alone (control) showed some cellular antioxidant protection in this assay. At the three lowest concentrations Alkazone[®] mineral drops showed higher antioxidant protection than saline alone. Neither product nor control reached the IC 50 line and therefore a CAP-e value was not achieved.

The following red data points indicate cell lysing. Cell lysing can happen at higher doses of test products that for various reasons are not well tolerated by the live cells. Lysing can be caused by unfavorable pH, salt concentration and other factors.

3.4. Cellular Antioxidant Protection Distilled Water

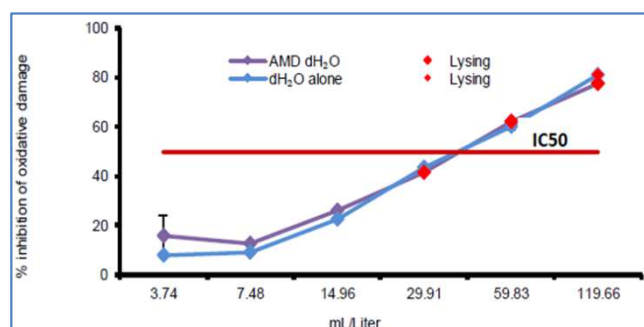


Figure 5. Cellular Antioxidant Protection with Alkazone[®] mineral drops in distilled water.

Both Alkazone[®] mineral drops in distilled water and distilled water alone (control) showed cellular antioxidant protection in this assay. At the three lowest concentrations Alkazone[®] mineral drops in distilled water showed higher antioxidant protection than distilled water alone. Both product and control reached the IC 50 line. The CAP-e value for the control was 4.5 CAP-e units per milliliter of test product. The CAP-e value for Alkazone[®] mineral drops in distilled water is an estimate only due to the fact that there was cellular lysing below and above the IC50 line. That estimate is 4.5 CAP-e units per milliliter of test product.

4. Conclusions

Alkazone[®] mineral drops had antioxidant effects when prepared following the instructions for human consumption. Alkazone[®] mineral drops showed antioxidant capacity in several tests.

Providing negative ORP readings both when dispersed in distilled water and in physiological saline. Providing a positive antioxidant capacity in the Folin-Ciocalteu assay. Alkazone[®] mineral drops showed cellular antioxidant protection from free radical stress. Providing a reduced cellular damage by free radicals in the CAP-e bioassay, using human red blood cells.

This project is an initial step in a planned series of laboratory testing and clinical pilot studies. Clinical pilot study on inflammation and free radical stress in humans; Clinical placebo-controlled trial will be conducted further.

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