Antioxidant Capability and Efficacy of Mega-H™ Silica Hydride, an Antioxidant Dietary Supplement, by In Vitro Cellular Analysis Using Photosensitization and Fluorescence Detection

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ABSTRACT

Treatment of Chinese hamster ovary and mouse hybridoma cells with Mega-H™ brand silica hydride, a marketed antioxidant, after photosensitization with singlet oxygen and hydroxyl/superoxide reactive oxygen species through the use of rose bengal diacetate and malachite green resulted in an effective method of reducing free radical activity by more than 96% against singlet oxygen species and more than 86% for hydroxyl and superoxide free radicals with the dosage recommended by the manufacturer. The analysis used a combinational spectralfluorometric technique to determine cell viability and cytotoxicity through the mechanism of intracellular esterase activity and plasma membrane integrity. Photosensitized controls not treated with silica hydride showed less than 1% viability under the same conditions. The reduction of the introduced free radicals and singlet oxygen species and the consequent high levels of cell viability may be the result of effective and efficient antioxidant and radical scavenging properties of silica hydride.

INTRODUCTION

The reactivity of biomolecules with free radicals and their relationship to oxidative stress have been the subject of a plethora of scientific investigations and consistently top the list of current topics in health and medicine. Reactive oxygen species (ROS) have been proven to cause numerous cellular anomalies, including but not limited to protein damage, deactivation of enzymatic activity, alteration of DNA, and lipid peroxidation of membranes. When the ROS accumulation exceeds the limits of what the natural cellular antioxidant effects can neutralize and render inert, numerous pathological effects may manifest in the cells, including atherosclerosis, senescence, carcinogenesis, reperfusion injury, rheumatoid arthritis, and various blood disorders. Additional oxidative stress–induced dysfunctions and diseases include cardiomyopathy, diabetes, porphyria, halogenated liver injury, Adriamycin cardiotoxicity, segmental progeria disorders, cataractogenesis, and multiple sclerosis. It has become evident over the last decade that ROS-induced pathologies are of a serious and significant nature.

In order to maintain cellular health, it is

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essential to have a specific and effective chemical scavenger to target multiple types of radicals. As healthy as one’s diet may be, environmental agents aid in the creation of ROS metabolic byproducts. Most commercially-based antioxidant supplements are not extraordinarily effective and target only a single oxidant.8,9 Because of the significant pathological implications of oxidative stress, it is important to find a specific scavenger to efficiently and effectively reduce multiple ROS. There is a balance in current commercially available antioxidants between safety for the consumer and effectiveness. Although most over-the-counter varieties of antioxidants are nontoxic at the recommended dosage, they also are not very effective reducing agents.10

In vitro radical scavenging assays have traditionally been performed by radical-inhibiting chemiluminescent or fluorometric analysis using a specific scavenger, or by techniques such as electron spin resonance. Results of some studies indicate that the pH of the solution has a significant impact on the efficacy of the antioxidant. An alkaline solution at about pH 8.5 has been shown to overtly increase the antioxidant effect by up to 60%, compared with the same compound tested in a near-biological pH of 7.4.11

Our assay used a series of simple techniques in fluorometric and photosensitization analysis to test the efficacy of a nonprescription product, silica hydride, marketed for its abilities as an antioxidant. Silica hydride is a novel silica-based polymeric colloid that contains interstitially placed hydride, H− ions. Preliminary laboratory and clinical studies showed silica hydride to be nontoxic and safe for consumption.12–14 The compound has exhibited unique characteristics, such as the ability to allow stable release of hydride ion in an aqueous environment for an extended period.15 This was demonstrated by simple proton nuclear magnetic resonance and ion-selective electrode analysis. The addition of silica hydride to water significantly reduces the potential by −700 mV, as measured by oxidation-reduction potential (ORP). Because ORP alone is not indicative of the true reducing power of a compound, owing to proton interactions resulting from changes in pH,16 a variation of the Nernst equation (Eq. 1) provides an effective means to measure the reducing potential for a compound that is reported in units of rH, a logarithmic scaled report denoting absolute reducing potential.

$$E_h = 1.23 - \frac{RT}{F}\ln \frac{1}{P_o}$$  \[Eq.1\]

where \(E_h\) is the measured ORP, \(F\) is the Faraday constant, \(R\) is the universal gas constant, and \(T\) is absolute temperature. The value 1.23 refers to the fact that the potential of oxygen under 1 atmosphere is 1.23 V greater than in a solution of the same pH. The rH is defined explicitly as the negative logarithm of the oxygen pressure, \(P_o\) (Eq. 2).

$$rH = -\log P_o$$  \[Eq.2\]

Silica hydride has been shown to have a reducing potential 6 rH units greater than that of vitamin C, ubiquinone (CoQ10), or β-carotene.

In addition to inducing a significant change in ORP, silica hydride raises the pH of an alkaline solution to about 8.7. This combination of reduction potential and pH makes for a very strong reducing agent that has numerous possibilities as an antioxidant and radical scavenger.

Photosensitization of cells has been well established and defined for the analysis of chemotherapeutic agents, cellular toxicity, and antioxidants, in addition to the in vivo and in vitro diagnosis and treatment of disorders.17–19 Traditionally, in antioxidant studies, agents such as 2,2'-azobis-2-aminopropane hydrochloride (AAPH) or cyanide-containing compounds such as sulfonated Zn-phthalocyanine and merocyanine 540 have been used to photosensitize plasma membranes.20–22 Photosensitizers allow for the quantitative as well as the qualitative analysis of the effectiveness and efficiency of reducing scavengers as antioxidants.22–26

We separately introduced several types of common, damaging radicals into Chinese hamster ovary (CHO) and mouse hybridoma (NS-1) cells. We then photosensitized them, invoking cytotoxicity, and then checked for cell viability and toxicity by using fluorescent probes designed to detect esterase activity in cellular membranes. The results objectively conveyed whether the cell was healthy and vi-
able, or dead and cytotoxic. Each photosensitized cell line was analyzed for cytotoxicity with and without treatment with silica hydride. The photosensitizing compounds, rose bengal diacetate (RBDA) and malachite green (MG), were used to introduce singlet oxygen and hydroxyl/superoxide ROS, respectively. The fluorescent probes used for quantification of cell viability provided a distinctive display depending on whether the cell was alive (bright green) or dead (red), using calcein AM and ethidium homodimer (EthD-1) as reagents.

Using animal cells as a human biological model, this study objectively and quantitatively illustrated the in vitro antioxidant properties of silica hydride.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and a potassium phosphate buffer solution (KH₂PO₄) were of analytical grade and were obtained from Sigma-Aldrich (Milwaukee, WI). The photosensitizers, RBDA and MG, and the viability and cell toxicity kit containing calcein AM and ethidium homodimer (EthD-1) as reagents, was from Molecular Probes (Eugene, OR). The silica hydride, sold as Mega-H™, was from Flantech Group (Soquel, CA).

Cell preparation

Five milliliters each of CHO and NS-1 cells were incubated at 37°C while the reagents were prepared. Each cell line sample was centrifuged at 100 g; the supernatant was removed and washed twice with a 50 mM KH₂PO₄ solution (pH 7.4). The final centrifuged pellets of each cell line were suspended in 3.0 ml of buffer. Five aliquots, of 1.0 ml each, from each cell line were pipetted into 2.0-ml centrifuge tubes. Twenty microliters of solution was placed on a hemocytometer and determined to contain 3 × 10⁴ cells per milliliter.

Singlet oxygen introduction

Under ultraviolet lighting, a 4 μM solution of RBDA was made in DMSO. One milliliter of the RBDA solution was added to two each of the previously prepared 1.0-ml aliquots for NS-1 and for CHO cells. The cells were allowed to stain at 23°C for 45 minutes. The stained cells were then washed twice in 50 mM KH₂PO₄.

Hydroxyl and superoxide introduction

Under ultraviolet lighting, a 4 μM solution of MG was made in DMSO and prepared with both cell lines as described for the singlet oxygen introduction.

Cell viability and cytotoxicity staining

To add the fluorescent viability probes to the cell lines, each of the four RBDA and MG suspensions for both NS-1 and CHO cells was centrifuged at 100 g and the supernatant was removed. The LIVE/DEAD mixture, consisting of 1.0 ml of a 2 μM calcein AM and 4 μM EthD-1 solution, was prepared as follows. Twenty microliters of the supplied stock EthD-1 solution was added to 10.0 ml of 50 mM PBS/50 mM KH₂PO₄ (pH 7.4), which was then added to each of the pellets. The solution was vortexed to ensure mixing. Five microliters of supplied stock calcein AM stock solution was then added, and the solution was further vortexed. The solution was allowed to stain each of the cell samples for 30 minutes at 23°C.

Control preparation

As a control, the final 1.0-ml aliquot from each of the original cell suspensions was stained with the LIVE/DEAD mixture as previously described. The cells in these solutions, however, were not photosensitized. Additional noncellular controls were prepared to substantiate that the silica hydride was not affecting fluorescence excitation or emission: 1 ml of 500 μg/ml aliquots of aqueous solutions of silica hydride prepared in double-distilled H₂O was combined with 1-ml solutions of 4 μM RBDA and of 4 μM MG. The excitation and emission spectra of both solutions remained the same as the spectra of the stock solutions of RBDA and MG. Similar controls were prepared and tested spectrally with the LIVE/DEAD calcein AM/EthD-1 mixture. The results showed no difference in excitation or emission spectra.
Silica hydride preparation

Aliquots of 500 μg silica hydride were added to one sample of RBDA-stained cells, one of MG-stained cells, and one control vial for each of the two cell lines and then vortexed for 30 seconds.

Experimental

On completion of the prepared cell suspensions, 150 μl of each of the samples was placed into a 150-μl sample quartz cuvette. All of the RBDA-stained samples were sensitized by treatment with a 543.5 nm helium neon laser (Melles Griot, Carlsbad, CA) for 30 minutes at 1,500 W/m². All samples of MG-stained cells were sensitized by treatment with a 632.8 nm helium neon laser (Melles Griot) for 30 minutes at 2,000 W/m².

Each of the samples was analyzed by a Jasco FP-750 spectrophotometer (Jasco Corp., Easton, MD) at 485 nm excitation and 530 nm emission, then viewed with fluorescence microscopy using an XF25 long-pass filter (Omega Optical, Brattleboro, VT). Spectra were taken and recorded to calculate the percent viability. Each assay was performed in six replicates. The experimental details are summarized in Table 1.

RESULTS

Nonfluorescent and cell-permeant calcein AM reacts with intracellular esterase to create a vibrantly fluorescent polyanionic calcein (530 nm emission) that is readily retained by live cells producing the esterase. Should the integrity of the cell membrane become compromised through cytotoxicity by photosensitization of the ROS, the EthD-1 enters the cell and esterase activity ceases, quenching the calcein emission and revealing the now fluorescent EthD-1 (645 nm emission) bound to nucleic acids. The absolute number of living cells is linearly related to the fluorescence signal obtained, and the percentage of live cells can be calculated by evaluating the quotient of the difference between sample and the maximum fluorescence, as illustrated by the equation (Eq. 3):

\[
\% \text{ Live Cells} = \frac{F(530)_{\text{sam}} - F(530)_{\text{min}}}{F(530)_{\text{max}} - F(530)_{\text{min}}} \times 100 \quad [\text{Eq. 3}]
\]

where \(F(530)_{\text{min}}\) is the fluorescence background noise, \(F(530)_{\text{sam}}\) is the sample reading, and \(F(530)_{\text{max}}\) is the maximum fluorescence reading. Reference spectra were taken of the stock calcein/EthD-1 solution to get a maximum fluorescence reading. Additional reference spectra were taken of the PBS/KH₂PO₄ buffer to obtain a background reading for the spectrophotometer.

The data obtained were calculated as a function of percent live cells. The results derived from the fluorometric analysis showed a 96.5% reduction of singlet oxygen ROS through the RBDA analysis; the reduction of hydroxyl and superoxide radicals was 86.3% effective. Figure 1A illustrates the viability after photosensitization of treated NS-1 and CHO cell lines intro-

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Cell type</th>
<th>Sensitizer</th>
<th>ROS</th>
<th>Silica hydride treated</th>
<th>Laser (nm)/time (min)</th>
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<tr>
<td>1</td>
<td>NS-1*</td>
<td>None</td>
<td>N/A</td>
<td>No</td>
<td>543.5/30</td>
</tr>
<tr>
<td>2</td>
<td>NS-1</td>
<td>RBDA</td>
<td>(^1\text{O}_2)</td>
<td>Yes</td>
<td>543.5/30</td>
</tr>
<tr>
<td>3</td>
<td>NS-1</td>
<td>RBDA</td>
<td>(^1\text{O}_2)</td>
<td>No</td>
<td>543.5/30</td>
</tr>
<tr>
<td>4</td>
<td>NS-1</td>
<td>MG</td>
<td>(\text{O}_2^- / \text{OH})</td>
<td>Yes</td>
<td>632.8/30</td>
</tr>
<tr>
<td>5</td>
<td>NS-1</td>
<td>MG</td>
<td>(\text{O}_2^- / \text{OH})</td>
<td>No</td>
<td>632.8/30</td>
</tr>
<tr>
<td>6</td>
<td>CHO*</td>
<td>None</td>
<td>N/A</td>
<td>No</td>
<td>543.5/30</td>
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<td>7</td>
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<td>RBDA</td>
<td>(^1\text{O}_2)</td>
<td>Yes</td>
<td>543.5/30</td>
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<td>RBDA</td>
<td>(^1\text{O}_2)</td>
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<tr>
<td>10</td>
<td>CHO</td>
<td>MG</td>
<td>(\text{O}_2^- / \text{OH})</td>
<td>No</td>
<td>632.8/30</td>
</tr>
</tbody>
</table>

*, Control.
duced to singlet oxygen radicals (RBDA) and hydroxyl/superoxide radicals (MG).

The viability rate of the nontreated photosensitized cells was 0.62%, as illustrated in Fig. 1B, showing that the cytotoxicity/death rate was consistent for both photosensitization techniques on both cell lines.

The controls cumulatively had a survival rate of 99.2% for the CHO cells and 99.1% for the NS-1 cells. Overall, the control survival rate

FIG. 1. (A) Percent viability of silica hydride–treated NS-1 and CHO cells as determined by calcein spectralfluorimetric analysis at 530 nm. CHO malachite green (—), NS-1 rose bengal (—), CHO rose bengal (—X—), and NS-1 malachite green (—) assays were performed in six replicates with consistent overall results, 83.3% ± 0.9% (S.D. 1.1) for malachite green and 96.5% ± 1.2% (S.D. 1.4) for rose bengal, cumulatively, for both cell types. (B) Percent viability of the photosensitized but not treated CHO and NS-1 cells. —, NS-1 rose bengal; —, CHO rose bengal; —, CHO malachite green; —X—, NS-1 malachite green. The overall viability statistics for rose bengal and malachite green are 0.73% ± 0.46% (S.D. 0.57) and 0.51% ± 0.31% (S.D. 0.40), respectively.
was 99.2% for both cell lines, (S.D., 0.7%), showing a consistent survival rate for both cell lines.

The overall data for the experiment are shown in Table 2.

### DISCUSSION

The use of silica hydride as a radical scavenger and antioxidant seems very promising with this in vitro analysis. Figure 1 provides significant evidence that the Mega-H™ silica hydride compound is able to efficiently reduce the radicals introduced into the cell lines. The doses of 500 μg/ml on a microscale are consistent with the doses suggested by the manufacturer of about 1.0 g/day for human consumption. The efficacy of the compound against singlet oxygen ROS appears to be greater, with less than a 3% loss of cell viability compared with controls. The effectiveness against hydroxyl and superoxide radicals also appears to be strong, with about 12% loss compared with controls. Perhaps the stoichiometric differences and nonspecificity of the MG to create only hydroxyl or only superoxide radicals accounts for a difference between the percentages. The efficiency of the silica hydride on each of the radical types did not significantly vary between cell lines, providing a basis to further hypothesize about the efficacy on all cell types. NS-1 and CHO are good indicators of how a compound might affect a human system.\(^{35-37}\) The silica hydride performed well in the reduction and elimination of oxidized species and in protection of the cells from oxidative stress.

Overall, the silica hydride exhibits significant properties. It has demonstrated in vitro reducing abilities, as measured by ORP. Additionally, it creates a slightly alkaline microenvironment with a relative hydrogen pressure (rH) value thousands of times greater than that of other tested antioxidants. As previously mentioned, the creation of an alkaline environment increases the efficacy of an antioxidant. Most marketed antioxidants actually create an acidic or neutral buffered environment.\(^6\) Cumulatively, silica hydride has many positive attributes as an effective antioxidant.

This simple but informative assay not only evaluated the ability of silica hydride to act as an antioxidant but also introduced the combinational techniques of using spectr fluorimetry with multiple fluorophores and photosensitization to quantify the efficacy of antioxidants and other reducing agents. This particular analysis used the calcein fluorescence in intact cells to obtain a percent viability. These techniques may be additionally used to calculate a percent dead/cytotoxic by evaluation of the fluorescence at 645 nm. These combinational techniques of fluorimetry, photosensitization, and viability testing may be used to perform simultaneous assays of different treatments on cells to show both percent viable and percent

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Cell type/agent</th>
<th>Treated</th>
<th>Percent viable (average %)</th>
<th>S.D.</th>
<th>±%</th>
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<td>1</td>
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<td></td>
<td>99.1</td>
<td>0.84</td>
<td>0.67</td>
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<td>2</td>
<td>NS-1/ RBDA</td>
<td>Yes</td>
<td>96.3</td>
<td>1.64</td>
<td>1.31</td>
</tr>
<tr>
<td>3</td>
<td>NS-1/ RBDA</td>
<td>No</td>
<td>0.62</td>
<td>0.60</td>
<td>0.48</td>
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<tr>
<td>4</td>
<td>NS-1/MG</td>
<td>Yes</td>
<td>86.3</td>
<td>1.44</td>
<td>1.16</td>
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<tr>
<td>5</td>
<td>NS-1/MG</td>
<td>No</td>
<td>0.67</td>
<td>0.46</td>
<td>0.37</td>
</tr>
<tr>
<td>6</td>
<td>CHO*</td>
<td></td>
<td>99.2</td>
<td>0.61</td>
<td>0.49</td>
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<tr>
<td>7</td>
<td>CHO/ RBDA</td>
<td>Yes</td>
<td>96.7</td>
<td>1.36</td>
<td>1.09</td>
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<tr>
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<td>CHO/ RBDA</td>
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<td>0.56</td>
<td>0.46</td>
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<tr>
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<td>Yes</td>
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<td>0.88</td>
<td>0.70</td>
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<td>CHO/MG</td>
<td>No</td>
<td>0.37</td>
<td>0.35</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*, Control.
dead, or to allow the testing of multiple ROS types in cells by a multiwavelength spectrophotometric and spectrofluorometric analysis.

In itself, the photosensitization assay performed provides significant evidence that silica hydride is an effective tool in the reduction of free radicals. Combined with the results of additional experimentation, the potential for the compound as an effective antioxidant is very great. We propose that the use of silica hydride may significantly reduce the oxidative stress and resultant pathologies induced by ROS in an efficient and effective manner. Additional studies are underway involving in vivo analysis of the compound on ROS to further validate or deny this hypothesis and to specifically quantify a minimum effective dose as well as determine the effectiveness of the compound against other radicals.

ACKNOWLEDGMENT

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REFERENCES

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