Interactions of Selenium Compounds with Other Antioxidants in DNA Damage and Apoptosis in Human Normal Keratinocytes

Chwan-Li Shen, Woosun Song, and Barbara C. Pence
Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Abstract
Selenium (Se) compounds are included in the human recommended daily allowance for Se intake is 55–70 μg/day, regardless of Se form, and the nontoxic dose limit for any Se supplement is considered 400 μg/day by the NIH Office of Dietary Supplements. For the purposes of this study, we have chosen to use 10 μg/ml (126.6 μM) Se as SeL and 25 μg/ml (316.6 μM) Se as SeM, but it is difficult to correlate these to plasma levels obtained from the various Se dietary supplements. Besides Se, other dietary antioxidants, such as Vit C or Vit

Introduction
Se is an essential dietary nutrient for all of mammalian species, including humans. The mechanisms of action of Se compounds, either via a pro-oxidant pathway, as seen in cytotoxicity and apoptosis, or via an antioxidant pathway as proposed in cancer chemoprevention, are still unclear but intriguing. Because the public may frequently supplement Se and other antioxidant compounds at high doses, the possible pro-oxidant effect of Se becomes a concern. Previous studies (1–6) have shown that SeL and its metabolites at high doses resulted in cytotoxicity, DNA fragmentation (7, 8), cellular apoptosis (1–4), and DNA oxidative damage in cells (6). The mechanism of the cytotoxicity of SeL and other redoxing Se compounds is believed to derive from its pro-oxidant ability to catalyze the oxidation of thiols and to produce superoxide simultaneously (5, 6). Our previous studies also support the findings that high doses of SeL supplementation resulted in cytotoxicity and induction of 8-OHdG lesions, an indicator of estimating DNA oxidative damage, in mouse skin cells (6) as well as in primary NHK (9).

Both SeL and SeM are two commercial forms of Se compounds that are available to and supplemented by the public. Our laboratory has shown previously that SeL and SeM have entirely different pharmacokinetic effects based on dose-related cytotoxicity in mouse skin cells (6) and in NHK (9). In general, on an equal Se dose basis, SeM is not as active as SeL, because SeM is a nonredoxing Se compound (3, 6). The mechanism of the cytotoxicity of SeL and other redoxing Se compounds is believed to derive from its pro-oxidant ability to catalyze the oxidation of thiols and to produce superoxide simultaneously (5, 6). Our previous studies also support the findings that high doses of SeL supplementation resulted in cytotoxicity and induction of 8-OHdG lesions, an indicator of estimating DNA oxidative damage, in mouse skin cells (6) as well as in primary NHK (9).
E are commonly supplemented and taken together with Se. Both Vit C and Vit E have been identified as antioxidant nutrients that may reduce the risk of cancer (10–12). Trolox, a commercial name for a water-soluble Vit E analogue, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, has been shown to protect mammalian cells from oxidative damage in vitro (13) as well as in vivo (14).

However, no studies have demonstrated the cellular interactions that would occur in cells supplemented with Se and other dietary antioxidants. The hypothesis to be tested in this study is that the antioxidants would interact with Se compounds and result in enhanced cellular protection. Thus, the present study was designed to investigate the cellular effects of SeL or SeM in combination with antioxidants (Vit C or trolox) by determining: (a) induction of cytotoxicity as measured by cell viability; and (b) generation of oxidative damage as seen in 8-OHdG lesions in DNA of NHK.

In addition, copper (II) is one of the major transition metals in the biological environment that is an active catalyst of DNA damage in vitro and in vivo (15). In general, cupric sulfate commonly presents in drinking water, soil, food, or the environment to increase the possibility of subchronic toxicity in humans (16). Interestingly, Davis et al. (2) have reported that copper as CuSO4 protects human colon cancer cells (HT-29) against SeL-induced cytotoxicity and apoptosis and acts as an antioxidant. In this study, we investigated the interaction between Se compounds and CuSO4 in NHK, as well as the interactive effects with Vit C and trolox.

Materials and Methods

Cell Culture. NHK (Cascade Biologicals, Portland, OR) were grown in culture at 37°C in 5% CO2/95% air using CM154 medium supplemented with 1% human keratinocyte growth supplement (Cascade Biologicals). Stock cells were plated and maintained under a culture density of 80% confluence (10^6 cells/100-mm plate) to minimize the proportion of cells mitotically arrested.

Treatment of NHK Cells. NHK were treated with no Se, SeL (126.6 μM Se), or SeM (316.6 μM Se) plus two doses each of Vit C (2.27 and 4.54 μM), trolox (40 and 80 μM), or CuSO4 (7.85 and 15.7 μM) for 24 h. The 24-h time point was chosen from unpublished preliminary data indicating this was the optimal length of incubation with Se compounds for determination of the three end points, cytotoxicity, apoptosis, and oxidative DNA damage. We chose two Se compounds (SeL and SeM) for their abilities to induce cytotoxicity and apoptosis. Therefore, higher doses of Se compounds were chosen to establish clear-cut end points that could be seen and modulated. The doses of SeL and SeM were determined by the limiting toxicity. Doses of Vit C, trolox, and Cu were chosen based on results with these compounds in our previous studies (2, 4). After 24 h, cells were detached with 0.025% trypsin/0.01 M EDTA and neutralized with trypsin neutralizer solution containing 0.5% calf serum (Cascade Biologicals).

Determination of Cytotoxicity. Cells were counted using a hemocytometer, and cell viability was measured by the method of trypan blue exclusion (1, 2). Cell pellets were resuspended in PBS and stored at −80°C for 8-OHdG analyses.

DNA Isolation and DNA Digestion. Cell DNA was isolated using a DNA purification kit (Qiagen, Valencia, CA). Cell DNA was enzymatically hydrolyzed according to a modified method adapted from Stewart et al. (4). Briefly, for 200 μl of DNA extract, 25 μl of 0.5 M sodium acetate (pH 5.1) was added along with 2.25 μl of 1 M magnesium chloride and mixed well. The DNA sample was boiled for 5 min and then placed on ice for 8 min. The DNA was digested to the nucleotide level by incubation with 1 unit of Nuclease P1 (Sigma, St. Louis, MO) at 37°C for 1 h. The DNA sample was optimized for alkaline phosphatase activity by adding 8 μl of 1 M Tris-base (pH 7.8). One unit of alkaline phosphatase (Sigma) was added to the DNA sample and incubated at 37°C for 1 h. Finally, 5 μl of glacial acetic acid:ddH2O (1:2, v/v) was added to the DNA sample. The DNA sample was filtered through a 0.2-μm syringe filter and prepared in an autosampler vial for HPLC injection.

Detection of 8-OHdG Adducts. The HPLC system consisted of a Waters Model 600 pump (Waters Associates, Milford, MA), a Waters Model 717 plus autosampler, a reverse-phase Rainin Microsorb-MV C18 column (4.6 mm × 25 cm; 5-μm particle size), a Waters Model 486 UV absorbance detector, a Waters amperometric Model 464 electrochemical detector with a glassy carbon electrode, and a Waters millennium chromatography software (version 2.0). The UV detector was equipped with a 254-nm lamp for the determination of 2-dG in DNA. A potential of 600 mV was applied to the electrochemical detector for the measurement of 8-OHdG adducts in DNA. The mobile phase was prepared containing 23 mM citric acid, 42 mM sodium acetate, 50 mM sodium hydroxide, and 1 ml/liter glacial acetic acid. The final running buffer was 20% mobile phase, 20% HPLC grade methanol, and 60% HPLC grade ddH2O, and the flow rate was 1.0 ml/min. Standards of 2-dG (Sigma) and 8-OHdG (ESA, Chelmsford, MA) were prepared in mobile phase. 2-dG and 8-OHdG standard curves were constructed based on peak heights and concentrations in a concentration-dependent manner and used for quantitation of 2-dG and 8-OHdG, respectively, in eluted DNA samples. The results were calculated based on the ratio of 8-OHdG (fmol):2-dG (fmol) and reported as numbers of 8-OHdG residues/10^6 2-dG residues.

Determination of DNA Fragmentation. DNA fragmentation assays were only performed on the combined supplementation with SeL and CuSO4 because previous results showed little DNA fragmentation with SeM, and cell viability was too low with SeL and trolox.

Cells were treated with SeL at doses of 0 and 126.6 μM Se plus CuSO4 (7.85 and 15.7 μM) for 24 h. Internucleosomal DNA fragmentation was measured by a cell death detection ELISAplus Kit (Boehringer Mannheim, Germany), as described previously (17). Briefly, after cells were treated with trypsin, cell pellets were resuspended in medium. The cytosplasmic fractions of a sample containing 10^4 cells were prepared following the manufacturer’s instructions. This assay is designed to measure the cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) contained in the cells that bind to an immobilized anti-histone antibody. Results were reported as a fragmentation ratio: the absorbance of the sample divided by the absorbance of the corresponding control (no Se and no CuSO4 added), indicating apoptosis as X-fold internucleosomal DNA fragmentation.

Statistical Analysis. The data were analyzed by one-way ANOVA, and mean separations were performed by Duncan’s multiple range test (P < 0.05). All of the analyses were performed on PC SAS (SAS Institute, Cary, NC).

Results

Cytotoxicity. Fig. 1, A–C, shows the viability of cultured NHK treated with SeL or SeM plus antioxidants or copper for 24 h. In general, compared with control (no Se or other compounds
In the absence of Se, supplementation of Vit C or trolox alone did not affect viability of NHK (Fig. 1, A and B). CuSO₄ alone appeared to decrease cell viability (Fig. 1C); however, this does not appear to be biologically relevant.

When NHK cells were coincubated with SeL at 126.6 μM Se and each of the other compounds, Vit C and CuSO₄ protected NHK against SeL-induced cytotoxicity (P < 0.05; Fig. 1, A and C). In contrast, the synergistic effect of SeL + trolox was found in the increased induction of cytotoxicity (P < 0.05; Fig. 1B), compared with supplementation of SeL alone. Regarding SeM plus other compounds, no changes in cell viability were observed in those treated with SeM + Vit C, SeM + trolox, or SeM + CuSO₄ shown in Fig. 1, A, B, and C, respectively.

**Induction of 8-OHdG Adduct Formation.** Fig. 2 demonstrates the formation of 8-OHdG adducts in DNA of NHK treated with SeL or SeM plus other compounds for 24 h. Compared with the control (no Se or other compounds), SeL alone elevated 8-OHdG induction in the DNA of human keratinocytes (P < 0.05), whereas SeM alone did not induce generation of 8-OHdG adducts.

Without Se supplementation, neither Vit C, trolox, nor CuSO₄ increased the formation of 8-OHdG adducts (P > 0.05; Fig. 2, A–C). Protection of NHK against SeL-induced DNA damage was observed in the treatments with SeL + Vit C and SeL + CuSO₄ (Fig. 2, A and C). However, SeL plus high dose trolox (80 μM) enhanced the formation of 8-OHdG adducts, compared with SeL added alone (Fig. 2B). When NHK cells were coincubated with SeM at 316.6 μM Se and either trolox (40 and 80 μM) or CuSO₄ (15.7 μM), there was increased induction of 8-OHdG adducts (Fig. 2, B and C).

**Protective Effect on SeL Induced by Copper.** In the present study, we only measured DNA fragmentation in NHK treated with SeL and CuSO₄, as discussed in a previous section. Because there was only minimal inhibition of SeL-induced 8-OHdG with Vit C or trolox, we did not perform the apoptosis assays with these compounds. Supplementation with SeL alone significantly increased internucleosomal DNA fragmentation (P < 0.05; Table 1). Supplementation of CuSO₄, even at a dose of 15.7 μM, did not cause DNA of cultured human keratinocytes to undergo cell apoptosis, as determined by internucleosomal DNA fragmentation (Table 1). Furthermore, the inhibition of SeL-induced apoptosis by CuSO₄ supplementation was observed.

**Discussion**

**Main Effects on Cytotoxicity by Se, Antioxidants, and Cu Alone.** Studies (5, 18, 19) have demonstrated that Se toxicity to cells results from increased thiol oxidation, redox cycling, and superoxide generation in cells in a Se concentration-dependent pattern. In the present study, we compared the effects of two commercially available Se compounds, SeL and SeM, on cytotoxicity to NHK. Our observations that SeL at a dose of 126.6 μM Se is cytotoxic to NHK are similar to those reported in other cultured cell studies (2, 20, 21), probably because of its capability of redox cycling and superoxide production. In the present study, the finding that SeM in NHK culture, even at a dose of 316.6 μM Se, did not cause DNA of cultured human keratinocytes to undergo cell apoptosis, as determined by internucleosomal DNA fragmentation (Table 1). Furthermore, the inhibition of SeL-induced apoptosis by CuSO₄ supplementation was observed.

Vits C and E have been represented as antioxidant nutrients that may reduce cancer risk (10–12). Vit E is the most important physiological membrane-associated lipid-soluble antioxidant in a biological model. Vit E has been reported to...
inhibit lipid peroxidation generated by dietary fat during the proliferation of various types of cells both *in vivo* (23, 24) and *in vitro* (25–28). In this study, instead of Vit E, we used water-soluble trolox (a Vit E analogue) acting as an antioxidant in the aqueous medium of cultured cells (4). As we expected, the antioxidants, Vit C and trolox, did not induce cytotoxicity in cultured NHK cells. Vit C and trolox have been shown to protect cultured mouse keratinocytes (4) as well as hairless mice (Skh:HR-1) skin (29) from UV B radiation-induced damage. We have also confirmed the ability of Se compounds to protect against UV-induced 8-OHdG in epidermis and incorporation into glutathione peroxidase in hairless mice.4

Copper (II) is one of the major transition metals in the biological environment (15). Because humans may be exposed to cupric sulfate through drinking water, food, soil, or the environment, a high risk of subchronic copper toxicity may occur (16). Copper (II) has been shown to catalyze DNA damage extensively both *in vitro* and *in vivo* (15). Our finding that CuSO₄ alone induced cytotoxicity in NHK cells confirms work from other laboratories (2, 15, 30) using different methodologies and cell lines.

**Interactive Effects between Se and Antioxidants/Cu on Cytotoxicity.** The present study was designed to investigate the cellular interactions between Se and antioxidants or CuSO₄ in a cultured NHK model. Trypan blue exclusion was used for determining cytotoxicity on the basis of cell viability. In the present study, Vit C partially protected NHK cells from SeL-induced cytotoxicity after 24 h of coincubation with SeL (Fig. 1A). However, our findings, as shown in Fig. 1B (SeL + trolox), demonstrated that trolox enhanced SeL-induced cytotoxicity of NHK cells. Although the mechanism by which trolox induces NHK cells to enhance SeL-induced apoptosis is still unknown, several studies have shown that Vit E increases the cytotoxic effects of antitumor drugs in both carcinoma cell cultures (31–35) and laboratory animals (31, 34). Additionally, Vit E not only acts as an antioxidant but may also intensify antineoplastic activity of anticancer drugs by inducing apoptosis (31, 36) through an inhibition of protein tyrosine kinases (36).

The observations that CuSO₄ protected NHK cells from SeL-induced cytotoxicity as illustrated in Fig. 1C are in agreement with those in (SeL + CuSO₄)-treated HT-29 cells (2). Davis and Spallholz (37) and others have suggested that Cu may complex with SeL to form a CuSe complex. The proposed complex of CuSe appears to inhibit the SeL-catalyzed generation of reactive oxygen species, which is known to induce apoptosis and DNA damage. In a cell-free model, copper (II) was shown to inhibit both SeL-catalyzed generation of superoxide and the conversion of SeL to elemental Se (37).

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*Fig. 2.* Numbers of 8-OHdG adducts/105 2-dG residues in DNA of NHK. Cells were treated with SeL or SeM plus Vit C (A), trolox (B), or CuSO₄ (C) for 24 h. Results are expressed as means; bars, ± SE; n = 3 for each treatment. Bars within Se doses sharing identical letters (a–d) are not statistically significantly different at P = 0.05 by Duncan’s multiple range test.

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Table 1 Effects of SeL plus copper (CuSO₄) on internucleosomal DNA fragmentation in DNA extracted from NHK cells.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>X-fold increase in apoptotic fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no Se, no CuSO₄)</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>SeL (126.6 μM Se) only</td>
<td>15.6 ± 0.5</td>
</tr>
<tr>
<td>CuSO₄ (7.85 μM) only</td>
<td>ND</td>
</tr>
<tr>
<td>CuSO₄ (15.7 μM) only</td>
<td>ND</td>
</tr>
<tr>
<td>SeL (126.6 μM Se) + CuSO₄ (7.85 μM)</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td>SeL (126.6 μM Se) + CuSO₄ (15.7 μM)</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

* Values are means ± SE, n = 3/treatment.
* Values with the same superscripts (b, c, d) are not significantly different P < 0.05.
* ND, not detectable.

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*Unpublished data.*
Our data showed that SeL at a dose of 126.6 compounds to induce oxidative stress in cultured NHK cells. We have compared the capability of two Se compounds. In the present study, we measured the number of 8-OHdG residues in vivo mouse skin cells (6).

Main Effects on DNA Oxidative Damage by Se, Antioxidants, or Cu Alone. The formation of 8-OHdG-adducted bases is an oxidative modification of DNA detected both in vivo (39) and in cultured cells (4, 15, 39, 40) and is considered as a consequence of oxidative stress (4, 39, 41). There is a strong correlation between higher amounts of 8-OHdG adducts and greater degrees of DNA strand breaks (15) or DNA damage (4). Several investigators have reported that high doses of SeL in in vitro studies resulted in DNA oxidative damage as determined by 8-OHdG lesions (4, 9), induction of cytotoxicity (2, 4, 9), and apoptosis as measured by DNA strand breaks or DNA ladders (1, 8, 9). In this study, we used the Floyd et al. (42, 43) method to measure 8-OHdG in keratinocyte DNA, and this method has consistently produced a mean of 0.5–1.5 8-OHdG/10^7 2dG in DNA in our control treatment (no Se as well or other compounds added). We have compared the capability of two Se compounds to induce oxidative stress in cultured NHK cells. Our data showed that SeL at a dose of 126.6 μM Se significantly induced oxidative DNA damage as 8-OHdG adducts in DNA of NHK, compared with the control (no Se added; Fig. 2). Levels of the 8-OHdG bases in NHK cells treated with SeL, were seen to be 15–20 times the number of 8-OHdG adducted bases in control NHK cells. SeM-treated NHK cells expressed no more 8-OHdG bases than did the control NHK cells. In agreement with previous findings (6), high dose SeL, acting as a pro-oxidant, induced cytotoxicity and DNA adducts in mouse skin cells, whereas SeM did not.

Interactive Effects on DNA Oxidative Damage between Se and Antioxidants/Cu. In the present study, we found Vit C or trolox alone did not induce DNA oxidative damage in NHK (Fig. 2, A and B). However, it is notable that coincubation of SeL with Vit C in medium for 24 h significantly reduced the levels of 8-OHdG, compared with those induced by SeL at a dose of 126.6 μM Se alone (Fig. 2A). Other studies (4, 44) have shown that the DNA oxidative damage, resulting as a consequence of UV irradiation, can be attenuated by supplementation of antioxidant nutrients (such as Vit C or trolox), resulting in a reduced number of 8-OHdG adducts in different cell lines. However, in the present study, we showed that both Se compounds (SeL and SeM) plus trolox significantly increased the amounts of 8-OHdG in DNA of NHK (Fig. 2B). Further investigation is needed to elucidate the interactions between Se compounds and water-soluble trolox on oxidative DNA damage during cell growth, possibly related to generation by Se of a Vit E radical enhancing the damage.

Interactive Effects on Apoptosis between Se and Copper. Metal ions, such as copper (II), act as a transition metal that can catalyze extensive DNA damage to induce the generation of oxidative 8-OHdG adducts in various in vitro models (15, 40). In this study, we measured the number of 8-OHdG residues after treating NHK cells with CuSO₄ alone for 24 h as shown in Fig. 2C. In our cultured NHK cells, CuSO₄ alone (even at a dose of 15.7 μM) did not enhance 8-OHdG formation, although this is not consistent with results reported previously (15) by others using supercoiled plasmid DNA.

In the present study, we chose only to measure apoptosis in NHK treated with SeL plus CuSO₄, because no significant interaction was seen in NHK treated with SeM plus antioxidants. In addition, the magnitude of differences seen in 8-OHdG generation by SeL + Vit C or trolox was not substantial enough to expect induction of apoptosis. In this study, SeL treatment caused a 15-fold increase in internucleosomal DNA fragmentation as measured by an ELISA kit, compared with the control (no Se added; Table 1). Supplementation of CuSO₄ alone did not cause cells to undergo apoptosis in our cultured NHK cells. However, compared with SeL supplementation alone, treatment of SeL + CuSO₄ resulted in higher cell viability (Fig. 1C), fewer 8-OHdG adducts (Fig. 2C), and a lesser degree of internucleosomal DNA fragmentation (Table 1). We have reported similar results with SeL and CuSO₄ in HT-29 cells (2) and in cell-free systems (37).

A summary of effects of intercellular interaction between Se and other antioxidant compounds is shown in Table 2. Our results confirm that cytotoxicity and oxidative DNA base modification are directly interrelated in cultured NHK. We have shown previously (9) a significant correlation between 8-OHdG formation, DNA strand breaks, and apoptosis by Se compounds. Our present data suggest that Vit C appears to scavenge some of the superoxide radicals generated by SeL metabolism, thus producing fewer 8-OHdG in NHK DNA, when compared with those treated with SeL alone. In terms of Se + trolox, it appears that SeL interacts with trolox synergistically to increase the pro-oxidant effects of SeL. We speculate that trolox may be functioning as a Vit E radical in conjunction with SeL. However, the possibility also exists that trolox may exert unknown indirect effects as well. Regarding SeL + CuSO₄, CuSO₄ appears to play a protective role in SeL-induced cytotoxicity, oxidative damage, and apoptosis of NHK, when compared with SeL alone. It is apparent from these data that significant interactions occur at the cellular and molecular level among antioxidant supplements; however, the impact from dietary combinations cannot be predicted from these in vitro studies. Additional studies are also needed to investigate the implications of these interactions for chemoprevention with SeL and possibly other Se compounds.

References

Table 2  Summary of effects of intercellular interaction between Se compounds and antioxidants a

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Selenium</th>
<th>Antioxidants</th>
<th>Cell viability</th>
<th>8-OHdG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no Se, no antioxidants)</td>
<td></td>
<td></td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>SeM</td>
<td></td>
<td>Vit C</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>SeM</td>
<td>Trolox</td>
<td>No change</td>
<td>↑ 7.6-fold</td>
<td></td>
</tr>
<tr>
<td>SeM</td>
<td>CuSO₄</td>
<td>No change</td>
<td>↑ 3.3-fold</td>
<td></td>
</tr>
<tr>
<td>SeL</td>
<td>0</td>
<td>↓ 48%</td>
<td>↑ 22.7-fold</td>
<td></td>
</tr>
<tr>
<td>SeL</td>
<td>Vit C</td>
<td>↓ 60%</td>
<td>↑ 14.5-fold</td>
<td></td>
</tr>
<tr>
<td>SeL</td>
<td>Trolox</td>
<td>↓ 20%</td>
<td>↑ 23.8-fold</td>
<td></td>
</tr>
<tr>
<td>SeL</td>
<td>CuSO₄</td>
<td>No change</td>
<td>No change</td>
<td></td>
</tr>
</tbody>
</table>

a The above information is based on the data from Figs. 1 and 2. All of the treatments are compared with the control (no Se, no antioxidants supplemented).


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