Damage to DNA by Cadmium or Nickel in the Presence of Ascorbate*

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ABSTRACT

The interactive effects of the anti-oxidant ascorbate (Asc) and the metals cadmium (Cd, as CdCl₂) or nickel (Ni, as NiCl₂) on the in vitro formation of breaks in double-stranded deoxyribonucleic acid (d/s DNA) were determined. Concentrations of 50 µM Cd or 200 µM Ni were dosed for 4 hours in factorial combinations with 500 µM Asc in RPMI 1640 medium (7 percent bovine serum) in which AHH-1 TK +/− cells (a spontaneously transformed human B lymphoblastoid cell line by Gentest Corp.) were replicating. In combination with Asc, Cd caused significant d/s DNA breaks (p < 0.01, n = 5), while Cd in the absence of Asc produced only a slight (but not significantly different) amount of d/s DNA damage when compared to the cells with no Cd added. The Asc alone was not damaging. The Cd caused damage to the d/s DNA only when Asc was present. The percent of d/s DNA remaining following the respective treatments was: +Cd + Asc, 13 ± 3; +Cd − Asc, 46 ± 8; −Cd + Asc, 54 ± 5; −Cd − Asc, 55 ± 7. Conversely, the presence of Ni resulted in increased amounts (percent) of d/s DNA compared to control values: +Ni + Asc, 63 ± 5; +Ni − Asc, 58 ± 5; −Ni + Asc, 52 ± 1; −Ni − Asc, 51 ± 4, (p < 0.05, n = 3). The contrasting results between Cd and Ni in the presence of Asc may reside in the point of action; while Cd acts directly on DNA, Ni is reported to act on heterochromatin. Although Asc is a recognized anti-oxidant, its presence in the media mixture potentiated d/s DNA damage from the Cd. This may be caused by a Fenton-type reaction in which an antioxidant in the presence of metal generates hydroxyl radicals and consequently d/s DNA breaks. Oxidative reactions between metals, oxygen, and antioxidants such as Asc may represent an important mechanism of cell death, toxicity, and transformation.

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Introduction

The carcinogenic and mutagenic processes are affected and influenced in various ways depending on interplay between risk factors, dietary constituents, and metabolic processes. For example, the respective interactions between the antioxidant Asc and heavy metals in vivo are different for interactions that occur under in vitro conditions. Shi et al. proposed that Asc with its antioxidant ability may provide a detoxification pathway in respect to radical generation on peroxynitrite decomposition under physiological conditions. Shiraishi et al. showed that pretreatment of rats with Asc prevented or significantly reduced cadmium-induced lethality. Mutagenicity of a number of carcinogens is inhibited in the Ames test by Asc. The Asc has a protective effect against carcinogenesis. Aidoo et al. showed that Asc intake of rats inhibits the mutagenicity of ethyl-nitrosourea. However, under in vitro conditions in which specific heavy metals are combined with Asc, damaging reactive oxidative products can be formed.

A balance between oxidation and antioxidation is constantly on-going and essential for the health of an organism. Free radicals are formed (among other ways) in normal cell metabolism and may even play an essential role in maintaining normal cell function. O$_2$-dependent organisms defend themselves with products of free radical biochemistry. The antioxidant ability of food nutrients such as Asc, vitamin E, and the flavonoids is being investigated to address their actual biological significance. Many of these antioxidant dietary micronutrients are considered to be cellular defense systems. Deficiencies in these micronutrients can weaken the defense and allow increased DNA damage. The presence or absence of cellular antioxidant defenses play a role in oxidant carcinogenesis. Under these circumstances, it would be expected that high antioxidant capacity would protect the DNA from oxidative damage and mutagenesis.

Cellular protection from oxidative damage involves many facets, including lipid and aqueous-phase antioxidants, as well as enzymatic systems such as glutathione peroxidase, superoxide dismutase, catalase, and a variety of DNA repair enzymes, which prevent or repair cellular oxidative damage. The role of antioxidant enzymes Cu, Zn-superoxide dismutase, catalase and glutathione peroxidase include protection from oxidative damage although the over expression of superoxide dismutase can sensitize rather than protect cells from oxidative stress.

The DNA strand breakage is an early event as a result of exposure of cells to oxidants. Damage to DNA occurs by both direct and indirect means, and both lead to cell death or mutagenesis. The exact mechanisms are not known. It is known that hydrogen peroxide (H$_2$O$_2$) forms hydroxyl radicals (·OH) intracellularly, which is usually the most likely free radical responsible for DNA damage. Free radicals, any chemical species capable of independent existence that possesses one or more unpaired electrons, are formed (among other ways) in normal cell metabolism and may even play an essential role in maintaining normal cell function. Endogenous processes leading to DNA damage include: (1) oxidation, (2) methylation, (3) deamination, and (4) depurination. Of these, oxidation appears to be the most significant. Damage to DNA probably contributes to the development of neoplasias by way of mutagenic or clastogenic effects. Oxidants are produced as by-products of mitochondrial electron transport, oxygen-utilizing systems, peroxisomes and other normal aerobic metabolism, and by lipid peroxi-
Oxidative stress often arises as a result of an imbalance in the body antioxidant status. Stimulated neutrophils and macrophages produce oxidants that induce cell injury, both in vivo and in vitro. Cellular metabolism generates reactive oxygen species, which include superoxide ions and H$_2$O$_2$. Trace metals, such as iron, can catalyze the formation of the hydroxyl radical (\(\cdot\)OH) from H$_2$O$_2$, which in turn causes DNA damage.

**Methods**

**Cells**

The lymphocyte cells used were AHH-1 TK+/-, which is a human B lymphoblastoid cell line that was spontaneously transformed. They were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 9 percent horse serum, 1 percent L-glutamine (200 mM), and 1 percent penicillin/streptomycin (v/v).

**Experimental Design**

Each analysis employed a 2 x 2 factorial design using ±Cd and ±Asc, yielding four experimental groups, i.e., +Cd +Asc, +Cd -Asc, -Cd +Asc, -Cd -Asc. The same combination was used for the Ni/Asc experiments by substituting the Ni for the Cd. Doses of Ni were 200 \(\mu\)M of NiCl$_2$ in the medium for 4 hours. The Cd was more toxic to the cells than Ni, therefore exposures to the cells were for 4 hours at 50 \(\mu\)M of CdCl$_2$. The Asc was added at a concentration of 500 \(\mu\)M. Each treatment combination was statistically compared by analysis of variance (ANOVA) using generalized linear models.

**DNA Analysis**

Damage to DNA in the form of strand breaks was analyzed as described by Birnboim and Jevcak and James and Yin. This procedure consisted of exposure of the cell lysates to alkaline solutions and measuring the rate of strand unwinding using the fluorescent dye that interacts only with double-stranded DNA (d-s DNA). When DNA is exposed to alkaline conditions, the rate of unwinding is directly related to the presence or amount of alkali-labile breaks (sites) in the DNA. The greater the DNA damage, the faster or greater the unwinding will occur. Therefore, a greater amount of d-s DNA remains in cells sustaining the least amount of damage.

Approximately 2 x 10$^7$ cells were removed from each medium treatment group, centrifuged for 10 minutes at 400 \(\times\) g, and suspended in 2 ml of buffered 0.25 M meso-inositol with 1 mM MgCl$_2$ at pH 7.2. This solution was divided between 9 borosilicate test tubes (triplicate samples) for each treatment group and analyzed as outlined in Birnboim and Jevcak. Briefly, the cells were exposed to alkaline conditions for 30 minutes 0°C with a subsequent incubation at 15°C for 30 minutes. The cells were neutralized, ethidium bromide was added, and the presence of d-s DNA was measured by fluorescence spectrophotometry (excitation 520 nm, emission 590 nm, band width 10 nm). The percent of d-s DNA was calculated by subtracting the fluorescence response of a blank sample (produced when the cell extract is sonicated and treated under alkali conditions which causes complete unwinding of the DNA) from that of a sample which
estimates the unwinding time divided by the sample representing the total d-s DNA.

Results

A combination of Cd and Asc was very toxic with respect to the occurrence of d/s DNA breaks. At the concentrations used in this study, Cd alone or Asc alone exhibited only a slight to moderate amount of d/s DNA damage when compared to the cells with neither Cd or Asc added. The percent of undamaged, or d/s DNA remaining following the respective treatments as shown in table I were +Cd+Asc, 13 ± 3; +Cd−Asc, 46 ± 8; −Cd+Asc, 54 ± 5; −Cd−Asc, 55 ± 7. The combinations of Ni and Asc did not result in a similar effect. The results were +Ni+Asc, 64 ± 5; +Ni−Asc, 58 ± 5; −Ni+Asc, 52 ± 1; −Ni−Asc, 51 ± 4 (table II).

The doses of Cd and Ni selected for this study were based on a previous study and were adjusted to show little or no DNA damage in order that the metal/Asc would not be overwhelmed by either of the single components. These previous studies also showed that Ni was much less toxic to the DNA than the Cd, and there is evidence that Ni acts on the heterochromatin, and, therefore, does not act directly on the DNA molecule. It was intended that with the use of two metals with apparent different targets of action, the specificity of the action of the Asc could be evaluated. In order to evaluate the lack of a response similar to that of Cd, the Ni concentration was elevated to 500 µM. At this concentration, there was evidence of a Ni/Asc interaction (table III). The percent d/s DNA remaining at this concentration was 48 percent as compared to 61 to 64 percent for the other treatment combinations. When a dose

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n = 5 *p < 0.01  
AHH−1 = human B lymphoblastoid cell line.

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n = 3  
AHH−1 = human B lymphoblastoid cell line.

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AHH−1 = human B lymphoblastoid cell line.
response was performed with Ni in the absence of Asc, there was essentially no response between 0 to 1000 μM (table IV).

**Discussion**

Although Asc is a recognized antioxidant that has shown protective effects in *in vivo* studies, its presence in the *in vitro* media mixture potentiated the d/s DNA damage from the CdCl₂. The increased CdCl₂-induced DNA strand breakage appears to be caused by the induction of a Fenton-type reaction which generates hydroxyl radicals. In this reaction, ·OH is produced from the oxidation of Asc which was probably responsible for the d/s DNA breaking action of the Asc. Oxidative reactions among metal, oxygen, and an anti-oxidant (Asc in this case) may represent an important mechanism of cell death and/or transformation. The Asc without other metals present was stable and produced no d/s DNA damage.

Other metal ions have been shown to express this same type of increase in oxidative potential. Asc with chromate can cause DNA strand breaks by generation of reactive oxidative substances during autoxidation of Asc. Kortenkamp and O’Brian⁸ showed that Asc in combination with chromate induced strand breaks in DNA. This damage, which is attributed to reactive intermediates generated during the reduction process, is catalyzed by traces of metal ions, namely Cu(II) and Fe(III). Shi et al¹⁶ found that oxidation resulted from the presence of sodium sulfite, nickel(II) subsulfite, and nickel subsulfide. Ding et al¹⁷ showed that the reduction of vanadate(V) to vanadate(IV) in the presence of Asc generated hydroxyl radicals by way of a Fenton-like reaction. Daniel et al¹⁸ detected the production of oxidant species, probably hydroxyl free radicals, in suspensions of crystalline silica. Silica was able to mediate DNA strand breakage *in vitro*. Stinson et al¹⁹ found that inorganic nickel chloride induced hepatic DNA strand breaks and postulated that the breakage may be caused by the induction of the Fenton reaction which generated hydroxyl radicals. Baader et al²⁰ showed that the presence of 1 mM of Asc released iron from ferritin *in vitro* and acted as a pro-oxidant.

Randerath et al²¹ showed the formation of hydroxylated derivatives of cytosine, thymine, adenine, and guanine in DNA were enhanced by the presence of Asc in the reaction mixture and their total amounts were similar to those of the major oxidation product of 8-hydroxy-2'-deoxyguanosine. In aqueous solution, superoxide radical can oxidize Asc. It can also reduce certain iron complexes such as cytochrome c.

Lefebvre and Pezaraf²² deduced that Asc acts as a biological reductant. They demonstrated that the reaction of soluble Cr(VI) with Asc in aqueous aerated medium produced highly electrophilic species of oxygen capable of oxidizing formate to carboxylate radicals. Hydroxyl radicals were not detected in the medium. When reducing agents other than Asc were used, the product ion of reactive oxidative substance from Cr(VI) was not Asc-dependent. The origin of
reactive oxidant substances is molecular O₂, not H₂O₂. Shi et al.²³ showed that incubation of Cr(VI) with Asc generated Cr(V), Cr(IV), and ascorbate-derived carbon-centered alkyl radicals, as well as formyl radicals. Electrophoretic assays showed that Asc-derived free radicals caused d/s DNA breaks. The Cr(V)-mediated Fenton-like reactions caused these d/s DNA breaks.

The Fenton reaction generates active forms of hydroxyl radicals from H₂O₂, DNA-bound iron, and a constant source of reducing equivalents, as shown in the following reaction:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH} + \text{OH}^- 
\]

H₂O₂ generates hydroxyl radicals in the presence of transition metal ions. To maintain an ongoing Fenton reaction, an electron source must be available to regenerate the reduced metal. Thiols, ascorbate, and superoxide have been used in vitro for this purpose.²⁴ Ljungman and Hanawalt²⁵ showed that binding of histones to the DNA and its organization into higher order chromatin structures protects the DNA from strand breaks from hydroxyl radicals produced by a Fenton reaction involving Fe(II), ethylenediamine tetraacetic acid (EDTA), and Asc. Goldstein et al.²⁶ showed that numerous transition metal ions and their complexes in their lower oxidation states were found to have the oxidative features of the Fenton reagent, and designated the mixtures of these metal compounds with H₂O₂ to be "Fenton-like" reagents. Daniel et al.¹⁸ detected the production of oxidant species in suspensions of crystalline silica in which both superoxide and Fenton reaction oxidants were produced, leading them to conclude that silica is able to mediate DNA strand breakage in vitro. Li et al.²⁷ demonstrated the DNA-breaking action of Asc in the presence of Cu(II), but indicated that the simple Fenton reaction could not account for all the breaking action on the DNA. Tadolini and Cabrini²⁸ showed that in the absence of OH scavengers, OH damage to deoxyribose is higher at acidic compared to neutral and moderately basic pH. At acidic pH, deoxyribose is able to inhibit Fe²⁺ oxidation by H₂O₂. Littlefield et al.²⁹ showed that other oxidative products were more likely to form at slightly acidic pH conditions.

Stearns et al.³⁰ conducted an investigation into the role of reactive intermediates with respect to the reduction of Cr(VI) by Asc and concluded that it is not yet understood whether the intracellular reduction of Cr(VI) by Asc is a detoxification process, i.e., does Asc protect tissues against Cr(VI)-induced DNA damage by inducing it to an inert form, Cr(III), or does this process produce reactive intermediates that are the actual genotoxic species. Stearns et al.³¹ also demonstrated in another study that reaction of Cr(VI) with varying Asc in calf thymus DNA resulted in Cr-DNA adducts and DNA strand breaks. Shi et al.³² showed that incubation of Cr(VI) with Asc generated Cr(V), Cr(IV), and Asc-derived carbon-centered alkyl radicals. The Asc-derived free radical caused d/s DNA breaks.

The oxidative potential of Asc has been manifested in other endpoints in addition to the Fenton reaction. Yoshimura et al.³³ showed that 8-hydroxydeoxyguanosine (8-OH-dG) was formed in deoxyguanosine solution by the addition of Fe²⁺, Asc, EDTA, and H₂O₂. Park and Floyd³⁴ showed that 8-OH-dG was formed in calf thymus DNA by a metal-catalyzed oxidation system composed of Fe₃, O₂, and a thiol as an electron donor.

Oxidative damage can originate from many conditions and sources. Cellular metabolism generates oxygen species, which may include superoxide anions and H₂O₂. Leukocytes release superoxide and H₂O₂ that can, in the presence of catalytic amounts of Fe, be converted into the potent oxidant hydroxyl radical
(·OH) and this oxidant induces DNA damage and strand breaks. This study shows that one of the principle sources of oxidants is from metal interactions. Under in vivo conditions, antioxidants such as Asc are supposed to defend against oxidative damage. Fraga et al showed that oxidative DNA damage was inversely correlated with Asc levels in seminal fluid, which in turn were related to the amount of dietary Asc consumed.

The Asc has long been recognized as a primary naturally occurring antioxidant. While it serves as a protectant in the intact biological system, there is much recent data to indicate that under in vitro conditions, Asc serves as a donor of reactive O₂ in the presence of heavy metals and potentiates the formation of damaging by-products. It has been estimated that there are as many as 10,000 of these oxidative hits on each cell per day. It is possible that metal-catalyzed Fenton reactions in the presence of Asc may be one of the most important paths of cellular DNA toxicity and carcinogenesis.

References


