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The Influence of High Doses of Vitamin C and Zinc on Oxidative DNA Damage

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Abstract. *Background: The influence of interactions between reactive oxygen species (ROS) and dietary antioxidants and their influence on cancer is not clear. It is believed that this effect is mediated by decreased oxidative damage to DNA. The aim of this study was to further investigate the in vitro DNA protective or damaging effects of dietary antioxidants using the single cell gel electrophoresis (Comet) assay. Materials and Methods: Stimulated and unstimulated lymphocytes of 10 individuals were cultured with and without different concentrations of vitamins C and zinc and damaged with H_2O_2. Results: DNA damage measured by Olive tail moment in the Comet assay showed a non-significant trend to reduce DNA strand breaks at low vitamin and trace element concentrations. At higher vitamin C and zinc doses, DNA damage was significantly increased. Conclusion: The in vitro data of the present study suggest that high dosage intake of vitamin C and zinc may cause more harm than benefit. There is good evidence that health-related effects of dietary antioxidants strongly depend on individual genetic susceptibilities and health status.*

Antioxidant protection against oxidant challenge may decrease the rate of mutation and hence help prevent ageing and age-related disease including cancer (1-5). Even though a balanced diet provides antioxidants, some people regularly take antioxidant supplements in the hope of preventing disease development by slowing down biological oxidative processes. In recent decades, high dosage supplementation of antioxidants has frequently been used in Western countries, and dietary antioxidants have also been implicated in chemoprevention trials for many cancer entities, including head and neck cancer (1).

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Malignant developments, such as head and neck cancer, are based on the occurrence of genetic alterations that result from an imbalance between DNA damage and repair. Oncogene activation and tumor suppressor gene inactivation caused by chromosomal alterations lead to uninhibited cell growth and tumor development (6-8). To date, little is known about interindividual differences in the general mutagen sensitivity of cancer patients compared to non-cancer patients.

The protective effect of fruits and vegetables against cancer is well established. It is believed that this effect is mediated by antioxidants (such as vitamin C and zinc) and decreased oxidative damage to DNA. Vitamin C is a strong, water soluble antioxidant and must be ingested in humans for survival. Human diseases such as atherosclerosis and cancer might occur in part from oxidant damage to proteins and DNA (4, 9, 10). Epidemiological studies have shown that diets high in fruits and vegetables are associated with lower risk of several diseases including cancer. Whether these protective effects are directly attributable to vitamin C is not known (2). As mean serum levels of vitamin C and zinc are significantly reduced in many cancer patients, particularly in those suffering from malnutrition as in head and neck cancer patients, these substances together with beta-carotene, alpha-tocopherol and retinoids are part of many chemoprevention trials (1). The influence of these dietary antioxidants and their interactions with reactive oxygen species (ROS) and the consequences of dietary antioxidant supplementation on cancer development in particular are not clear.

The aim of this study was to further investigate the *in vitro* DNA protective or damaging effects of dietary antioxidants (vitamin C and zinc) using the single cell gel electrophoresis (Comet) assay.

Materials and Methods

Preparation, harvesting and treatment of lymphocytes. Ethical approval for this study was granted by the Ethics Subcommittee of The Ludwig Maximilians University, Germany. Lymphocytes from fresh heparinized venous blood of 10 healthy subjects were used. Cryopreservation of

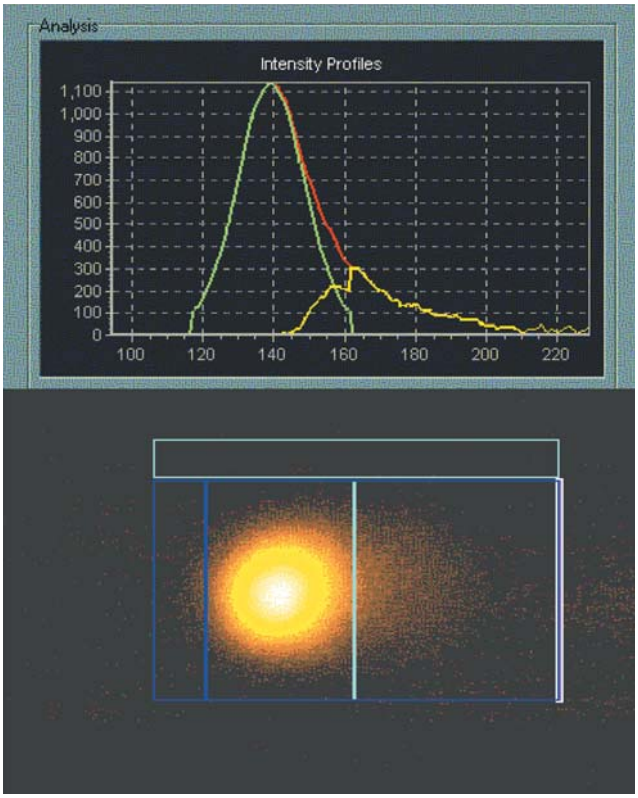

Figure 1. *Digital determination of the Olive tail moment (OTM: median DNA migration distance x relative amount of DNA in the tail of the comet).*

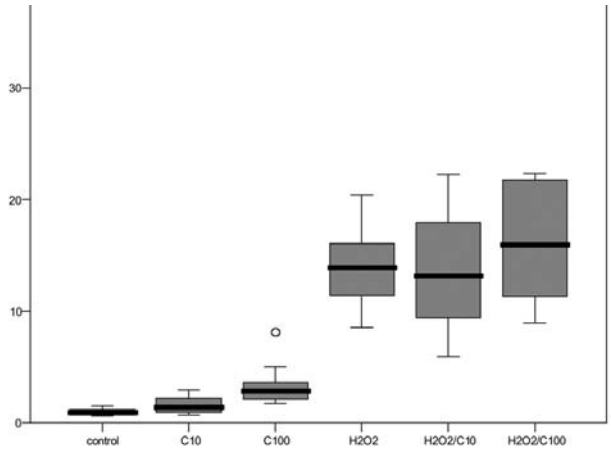

Figure 2. *Comet assay OTM of lymphocytes coincubated with vitamin C 10 μM / 100 μM, damaged with H_2O_2.*

lymphocytes was performed according to the procedure previously published (8, 11, 15). Stimulated lymphocytes were incubated for 25 h with 100 μl phythemagglutinine for 5,000,000 cells.

Comet assay. Lymphocytes underwent enzymic digestion (10 mg hyaluronidase, 10 mg collagenase, 50 mg protease) for 45 min at 37˚C

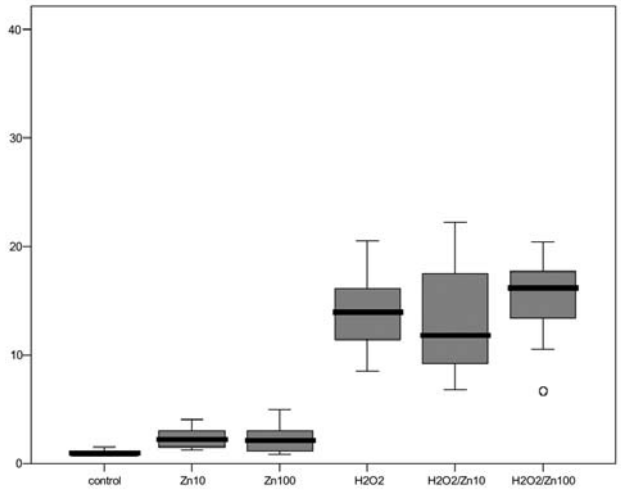

Figure 3. *Comet assay OTM of lymphocytes coincubated with zinc 10 μM / 100 μM, damaged with H_2O_2.*

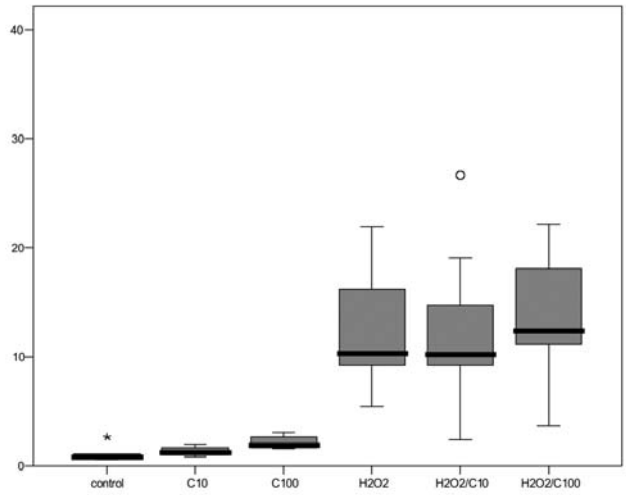

Figure 4. *Comet assay OTM of lymphocytes pretreated with vitamin C 10 μM / 100 μM, then damaged with H_2O_2.*

(11). Cell counts were made, and viability was tested with trypan blue staining. Cell aliquots of 1 ml (10.000 cells/ml) were treated with vitamin C and zinc at concentrations of 10 μmol and 100 μmol, washed in PBS and then incubated for 60 min with H_2O_2 (2.5 μmol).

The cells were resuspended in 0.7% low-melting agarose and applied onto slides, frosted at the long edges and covered with 0.5% normal melting agarosis, to provide stability of the agarosis layers. The slides were placed into a solution with 10% DMSO, 1% Triton-X, 2.5 M NaCl, 10 mM Trizma-Base, 100 mM Na_2EDTA and 1% N-lauroylsarcosine sodium salt for 1h. The slides were placed into a horizontal gel electrophoresis chamber (Renner, Dannstadt, Germany), positioned close to the anode and covered with alkaline buffer solution containing 300 mM NaOH and 1 mM

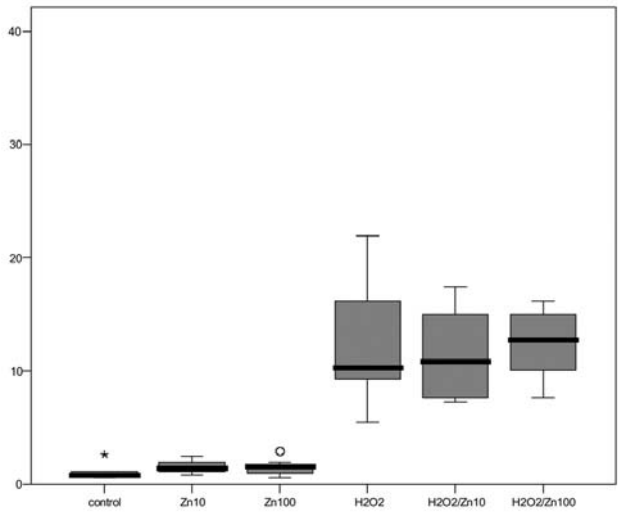

Figure 5. *Comet assay OTM of lymphocytes pretreated with zinc C 10 μM / 100 μM, then damaged with H_2O_2.*

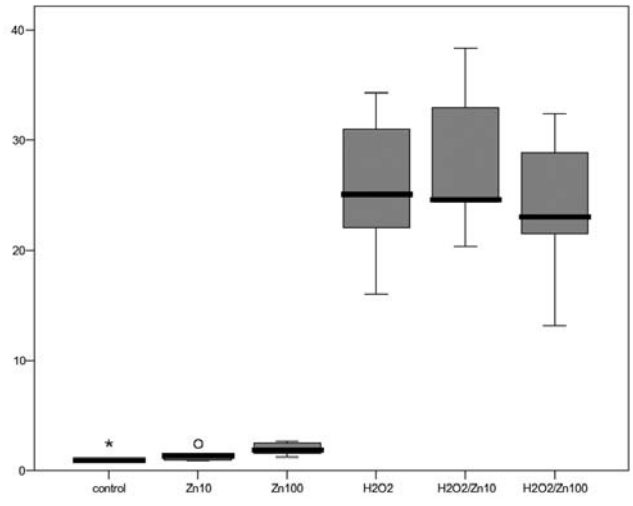

Figure 7. *Comet assay OTM of lymphocytes stimulated with PHA, then coincubated with zinc C 10 μM /100 μM and H_2O_2.*

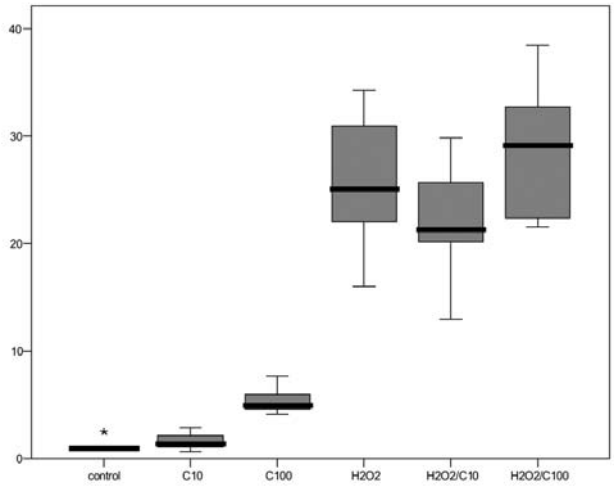

Figure 6. *Comet assay OTM of lymphocytes stimulated with PHA, then coincubated with vitamin C 10 μM /100 μM and H_2O_2.*

Na_2EDTA at pH 13.2. After a 20-min DNA "unwinding" period, electrophoresis was initiated at 0.8 V/cm and 300 mA for 20 min, followed by neutralization (400 mM Trizma base, pH 7.5; Merck).

Staining and analysis. Ten μl DAPI (42 ng/ml) with antifade were applied after air drying of the slides followed by storage of the slides at –20˚C protected from light. DNA fragmentation was visualized using a fluorescence microscope and digital analysis (Comet++, Kinetic Imaging™) (12) (Figure 1). Twenty cells per slide were analyzed.

Analysis of data. Statistical analysis was performed using SPSS 12.0™. OTM values of all groups were compared (Mann-Whitney *U*-test, Wilcoxon-test), and the general level of significance was $p \leq 0.05$. Results giving *p*-values up to 0.10 are listed for trends. *P*-values higher than 0.10 are classified not significant (n.s.).

Results

The results obtained using the Comet assay are shown in Figures 3 to 7. Statistical analysis is presented in Table I. Lymphocytes pretreated with antioxidants and damaged afterwards with H_2O_2 showed the lowest amount of DNA fragmentation, reflected by mean OTMs around 10-12. (Figures 3, 4). PHA-stimulated lymphocytes appear to be the most vulnerable with mean OTMs around 25 (Figures 5, 6), while untreated lymphocytes gave OTMs around 15 (Figures 1, 2). OTM data for single incubation with vitamin C and zinc as well as the coincubation with H_2O_2 were very similar in the differently-treated lymphocytes. PHA-stimulated lymphocytes showed similar DNA fragmentation patterns compared with unstimulated lymphocytes.

Incubation of lymphocytes with vitamin C and zinc alone resulted in a significant increase in OTMs compared with controls. This DNA fragmentation was dose-dependent, with higher OTMs at higher vitamin C and zinc dosages.

As expected, strongly increased tail DNA content, reflected by high OTMs, was found after treatment with H_2O_2. OTMs were only slightly reduced when coincubated with vitamin C 10 μM / H_2O_2 and zinc 10 μM / H_2O_2 compared with controls. Decreased damage is associated with decreased tail DNA content (reduced OTMs) and indicates a protective effect of antioxidant pre-treatment.

By contrast, coincubation with vitamin C 100 μM / H_2O_2 and zinc 100 μM / H_2O_2 resulted in a trend of increasing OTM.

Table I. *Statistical analysis of groups presented in Figures 2 to 7.*

Variable	*p*-value
Lymphocytes unstimulated, coincubated	
Vitamin C 10 μM *vs.* controls	0.028
Vitamin C 100 μM *vs.* controls	0.005
Zinc 10 μM *vs.* controls	0.007
Zinc 100 μM *vs.* controls	0.007
H_2O_2 *vs.* controls$_2$	<0.001
H_2O_2 and vitamin C 10 μM *vs.* H_2O_2	n.s.
H_2O_2 and vitamin C 100 μM *vs.* H_2O_2	n.s.
H_2O_2 and zinc 10 μM *vs.* H_2O_2	n.s.
H_2O_2 and zinc 100 μM *vs.* H_2O_2	n.s.
Lymphocytes unstimulated, pretreated with antioxidants	
Vitamin C 10 μM *vs.* controls	0.037
Vitamin C 100 μM *vs.* controls	0.007
Zinc 10 μM *vs.* controls	0.074
Zinc 100 μM *vs.* controls	n.s.
H_2O_2 *vs.* controls$_2$	<0.001
H_2O_2 and vitamin C 10 μM *vs.* H_2O_2	n.s.
H_2O_2 and vitamin C 100 μM *vs.* H_2O_2	n.s.
H_2O_2 and zinc 10 μM *vs.* H_2O_2	n.s.
H_2O_2 and zinc 100 μM *vs.* H_2O_2	n.s.
Lymphocytes stimulated, coincubated	
Vitamin C 10 μM *vs.* controls	n.s.
Vitamin C 100 μM *vs.* controls	0.043
Zinc 10 μM *vs.* controls	n.s.
Zinc 100 μM *vs.* controls	0.078
H_2O_2 *vs.* controls$_2$	<0.001
H_2O_2 and vitamin C 10 μM *vs.* H_2O_2	0.080
H_2O_2 and vitamin C 100 μM *vs.* H_2O_2	n.s.
H_2O_2 and zinc 10 μM *vs.* H_2O_2	n.s.
H_2O_2 and zinc 100 μM *vs.* H_2O_2	n.s.

Discussion

Numerous authors have described the benefits of a diet rich in fruit and vegetables, but the identity of the responsible substances is not clear (1-5). In particular, patients suffering from malnutrition (*i.e.* many head and neck cancer patients) seem to benefit from dietary antioxidants (2).

Genetic changes are dependent on exogenous and endogenous factors (6). Exogenous factors are xenobiotics and physical as well as biological hazards. They all can cause DNA strand breaks, which again lead to chromosomal aberrations and non-homologous recombination, resulting in apoptosis or modification of cell differentiation (13). The single cell gel electrophoresis, or 'Comet' assay is a well validated test for detecting DNA strand breaks in single cells (8, 13-16). Loops of DNA-containing breaks are pulled out of the nucleus in the direction of the anode, forming a 'comet tail'. The relative density of DNA in the tail is related to the degree of DNA damage (13).

In the present study, DNA damage in genotoxically-stressed cells was not reduced at all antioxidant concentrations tested. In fact, vitamin C and zinc appeared to increase the damage at higher concentrations. This effect was stronger when incubated with vitamin C and zinc alone compared to coincubation with H_2O_2. Thus, it can be concluded that antioxidants are only beneficial when oxidative stress is present. This strongly contradicts previously published linear inverse correlations between DNA strand breaks and vitamin C levels (5). The conclusion drawn by these investigators, that high dosage vitamin C intake is strongly protective against cancer, might only be true in certain populations.

Some studies have given evidence that DNA in human lymphocytes might be more resistant to oxidant challenge following antioxidant pre-treatment of the cells (3, 4). However, the influence of interactions between reactive oxygen species (ROS) and dietary antioxidants and the consequences of dietary antioxidant supplementation on human health and cancer development in particular are not clear. Dietary antioxidants take part in cellular reduction-oxidation reactions. Depending on the physiological environment, they can act as antioxidants (electron donors) or prooxidants (electron acceptors). Even though ROS are normal byproducts of mitochondrial aerobic metabolism and are essential for various cell defence mechanisms, they can cause oxidative DNA damage, resulting in increased cancer risk. In contrast, the possibility exists that in a pro-oxidant environment, in which desired levels of ROS interfere with dietary antioxidants, risk for cancer development may increase. There are various intracellular antioxidant mechanisms of DNA protection, including ROS scavenging, enzymatic inactivation of ROS and iron binding (9). Activation and altered transcription of antioxidant and DNA repair enzymes are also important to maintain low baseline levels of DNA damage under the normal intracellular conditions of continuous oxidant challenge of varying intensity.

Several studies using the Comet assay have shown that some antioxidants are indeed protective against oxidant challenge, but some appear to have no effect, and others may have a prooxidant, genotoxic effect under certain circumstances (3, 4, 10). Clearly, further study of the mechanism of action of putative antioxidants on DNA is needed.

Since antioxidants in the cellular environment do not necessarily exert the same effect as in cancer patients (due to these patients' specific dietary and often smoking habits), the results of the present study must be very carefully applied to particular patient groups. Although, dietary antioxidants as well as endogenous antioxidant mechanisms might help maintain the balance between the desirable and undesirable effects of ROS, the findings of our study

underline that dietary antioxidants are likely to be beneficial only at certain concentrations. These concentrations will strongly depend on the oxidative stress present in the *in vivo* situation, which may vary with different cancer entities and populations and, furthermore, may individually be influenced by genetic variation (*i.e.* at gene loci such as gluatathione S-transferase and cytochrome P450) (17, 18). A strong reduction in lung cancer risk has been found in heavy smoking men carrying the glutathione S-transferase M1 null genotype when their diet was supplemented with vitamin E compared to those patients with unsupplemented diets (19). Furthermore, different cancer entities may present different results. Accordingly, high dosage intake of a combination of 6 vitamins in non-metastatic breast cancer patients resulted in a shorter disease-free survival time compaired to matched controls (20).

Ideally, future studies of the antioxidant action of vitamin C and zinc should target selected patient groups. These groups should be known to have increased oxidative damage as assessed by a reliable biomarker. Our study gives evidence that high dosage intake of antioxidants, particularly in non-cancer patients with good nutrition habits and a diet rich in friuts and vegetables, may cause more harm than good.

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