Reduction of Oxidative DNA Fragmentation by Ascorbic Acid, Zinc and *N*-Acetylcysteine in Nasal Mucosa Tissue Cultures

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Abstract. *Oxidative stress is one major factor in upper aerodigestive tract carcinogenesis. Reactive oxygen species derived from environmental sources, cigarette smoke or cellular metabolism, constantly attack large molecules within the cell. While damaged lipids and proteins can be replaced, oxidative DNA damage needs to be repaired. Damage exceeding DNA repair capacity might lead to permanent mutations. Ascorbic acid, zinc and N-acetylcysteine are widely used as supplementations during upper aerodigestive tract infections. Therefore we chose to investigate their potential in DNA protection. We produced so called "mini-organ" cultures of nasal mucosa, three-dimensional tissue cubes coated with ciliated epithelium, for repeated incubation with ascorbic acid, zinc and N-acetylcysteine at different concentrations. This model has several advantages with respect to repeated incubations, metabolic competence of cells and standardized conditions compared to cell line experiments or animal models. After washing twice, oxidative damage was induced by hydrogen peroxide. Resulting DNA fragmentation was analyzed using the FPG-comet assay, a special modification of the alkaline single-cell microgel electrophoresis for the detection of the most prevalent oxidative DNA base modification. DNA damage was reduced within a range of 45-60%. Cell viability after incubations with hydrogenperoxide was >90%. Our results show strong DNA protective effects of the substances tested in accordance with epidemiological studies linking a diet rich in antioxidative micronutrients with a lowered risk for cancer development. The reasons for the failure of large antioxidant supplementation interventional trials need to be further investigated.*

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The upper aerodigestive tract is continuously exposed to chemicals that impose extensive oxidative stress on the human mucosa. Reactive oxygen species (ROS) derived from environmental sources such as cigarette smoke, or produced by cellular metabolism, *e.g.* during inflammation, may exceed the antioxidant capacity of the cell. First-line cellular targets of ROS are large molecules such as the plasma membrane, proteins in the cytoplasm as well as nuclear DNA. Whereas oxidatively damaged lipids and proteins are replaced during scheduled turnover, DNA lesions need to be repaired. DNA damage exceeding DNA repair can lead to permanent mutations. Furthermore, the repair capacity might be restricted due to oxidative lesions of the enzymes involved (1).

Normal rate generation of reactive species is associated with age-related cancer. Besides steadily undermining DNA stability, oxidative stress was also shown to suppress apoptosis, promote proliferation and to enhance invasiveness and metastasis of cancer (1). ROS were shown to modulate DNA methylation patterns and therefore gene expression during carcinogenesis, influencing transcription factors such as hypoxia-inducible factor-1, the key mediator in controlling genes involved in cell cycle control, inhibition of apoptosis and angiogenesis. ROS alter cellular signal transduction such as the phosphatidylinositol-3-kinase/Akt pathway, which was shown to be redox regulated and to modulate cell survival (2). Current estimations based on epidemiological studies associate about 30% of all cancer deaths with dietary habits (3). The antioxidant hypothesis, proposes that "vitamin C, vitamin E, carotenoids and other antioxidant nutrients afford protection against chronic diseases by decreasing oxidative damage" (4) and is supported by compelling epidemiological data, where a diet rich in fruits and vegetables is associated with a reduced risk for cancer development. This led to high expectations of the

beneficial effects regarding antioxidant supplementation, but despite all assumptions, clinical interventional trials failed to show cancer preventive effects of supplemented antioxidants. The α-Tocopherol, β-Carotene (ATBC) Cancer Prevention study, including more than 29,000 male smokers at the age of 50-69 years, actually showed an increased risk of lung cancer development to be associated with β-carotene supplementation after a 5- to 8-year follow-up (5). In recent years, the scientific community has tried to find answers as to why these studies failed to show any benefit. One contributing factor, certainly, is that the working hypothesis behind the studies was based on epidemiological data only (6). Regarding DNA damage, antioxidant supplementation often failed to significantly decrease levels of oxidative damage *in vivo* (7). Regarding ascorbic acid, somewhat contradictory results were seen in *in vitro* models (8). In our laboratory, we have established and employed a model of mini-organ cultures (MOC) for the detection of DNA-damaging agents with respect to upper aerodigestive tract cells (9). Most upper aerodigestive tract inflammations are caused by viral infections. As antibiotics are not capable of treating these infections, many patients use supplementary medication to help fight the inflammation. Ascorbic acid (vitamin *C*, Vit. C), zinc and *N*-acetylcysteine (NAC) as widely used supplements in this context. For the study presented here, we investigated the effect of these compounds on oxidatively induced DNA fragmentation in nasal mucosa tissue cultures.

Materials and Methods

Preparation of mini-organ cultures (MOC). The study was approved by the Ethics Committee of Ludwig Maximilians University, Munich, Germany (project 349-05). All biopsy donors were informed by the investigators and signed an informed consent statement. Mucosa samples were collected during surgery of the lower nasal turbinates. Specimens were dissected into cubes of 1 mm^3 excluding deeper layers and washed three times in bronchial epithelial cell basal medium (BEGM; Promocell, Heidelberg, Germany). Cubes were placed in 24-well plates, one in each well, and coated with 0.75% Agar Noble (Difco, Detroit, MI, USA) then dissolved in Dulbecco's modified Eagle's medium (Gibco, Eggenstein, Germany), 10% fetal calf serum (Gibco), non essential amino acids (Gibco) and amphotericin B (Gibco). BEGM was replaced every second day during cultivation After 7 days in 250 μl BEGM at 37.5˚C, 5% CO_2 and 100% relative humidity, MOC were completely coated with ciliated epithelium. On day 7, multiwell plates were changed to avoid adherence (9).

Preincubation. MOC were incubated with Vit. C (10 μM/100 μM; Sigma, Munich, Germany), zinc chloride (10 μM/ 100 μM; Merck, Darmstadt, Germany), or NAC (10 μM/100 μM; Sigma) on 3 consecutive days for 30 minutes. For the assessment of possible synergistic effects of Vit. C and NAC, both substances (10 μM each) were simultaneously added during a 30-minute incubation period. After each incubation, the growth medium was renewed twice. Following the last incubation, oxidative DNA damage was introduced with hydrogen peroxide (H_2O_2, 200 μM) within 30 minutes of incubation. Aqua bidest was used as the negative control in all experiments.

Alkaline single cell microgel electrophoresis (comet) assay. MOC were digested enzymatically thus gaining single cells. The enzyme suspension included collagenase P (1 mg/ml; Boehringer, Mannheim, Germany), hyaluronidase (1 mg/ml; Boehringer) and protease (5 mg/ml; Sigma). MOC were suspended for 60 minutes at 37.5˚C. After neutralizing histolytic enzymes with fetal calf serum (Gibco), single cells were washed twice in cold phosphate-buffered saline (Gibco), followed by trypan blue exclusion test to monitor cell viability.

Alkaline single-cell microgel electrophoresis (comet assay) was carried out according to the standard protocol (10). In order to increase the ability to detect oxidatively induced DNA damage, the enzyme formamidopyrimidine glycosylase (FPG; Boehringer) was used. FPG specifically recognizes 8-hydroxy-guanine, the most prevalent and potentially mutagenic oxidative DNA modification. FPG hydrolyses 8-hydroxy-guanine, leaving behind an apurinic side where, in the alkaline milieu of the comet assay, a single DNA strand break will be introduced, thus increasing DNA fragmentation (10). After lysis of cell and nuclear membranes, the slides were covered and incubated with a solution containing FPG.

DNA migration was measured using the image analysis software Komet 3.1 (Kinetic Imaging, Liverpool, UK) and quantified by the Olive tail moment (OTM). The OTM is the product of the relative amount of DNA in the tail and the median migration distance in micrometers (11). A total of 80 cell nuclei per slide were randomly selected without knowledge of pretreatment.

Statistical analysis. Significant differences in DNA damage between the samples were calculated by the Wilcoxon test using SPSS 16.0 software (SPSS GmbH, Munich, Germany). Calculation was based on the arithmetic mean of the OTMs of each slide, with the alpha level set at 0.05 prior to statistical analysis.

Results

Cell viability after incubations was >90% in all experiments.

The average OTM of negative controls was 1.5 (n=40). Vit. C, zinc chloride, NAC and the combination of Vit. C and NAC (each concentration; n=20) caused an average OTM <2. An OTM of 2 is the generally accepted threshold level, OTM >2 reflects considerable DNA fragmentation (10). H_2O_2 induced an average OTM of 7.9 (n=40). When MOC were incubated with Vit. C (10 μM, 100 μM) on 3 consecutive days, H_2O_2-induced DNA fragmentation (8.1 in this test series) was reduced to 3.6 and 3.2, respectively ($p<0.001$). Zincchloride (10 μM, 100 μM) reduced OTM to 3.5 and 3.2 ($p<0.001$; Figure 1). Preincubation of MOC with NAC (10 μ, 100 μM) lowered H_2O_2 induced DNA fragmentation (7.6 in this test series) by 45% and 47% to 4.2 and 3.9, respectively ($p<0.001$). These results are shown in Figure 2. The combination of Vit. C (10 μM) and NAC (10 μM) did not significantly change the reduction of DNA damage that was seen after incubation with these substances alone (see Figure 2).

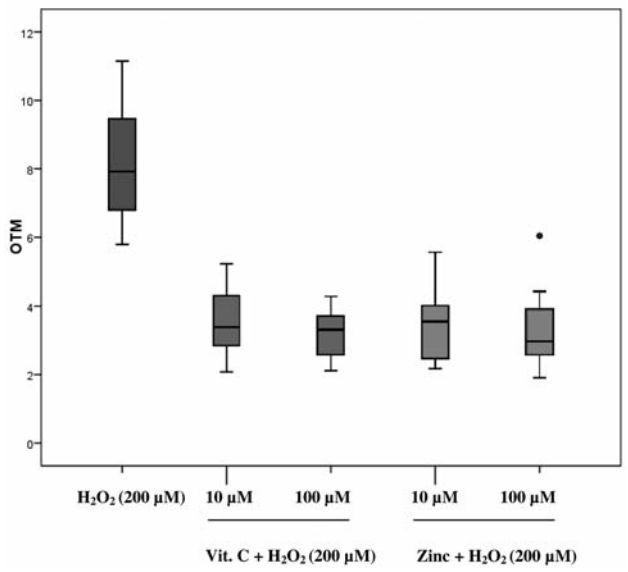

Figure 1. *Boxplot showing the reduction of H_2O_2-induced DNA fragmentation by ascorbic acid (Vit. C) and zinc chloride (Zinc) (n=20). *Outlier: value more than 1.5 and less than 3 box-lengths from end of box.*

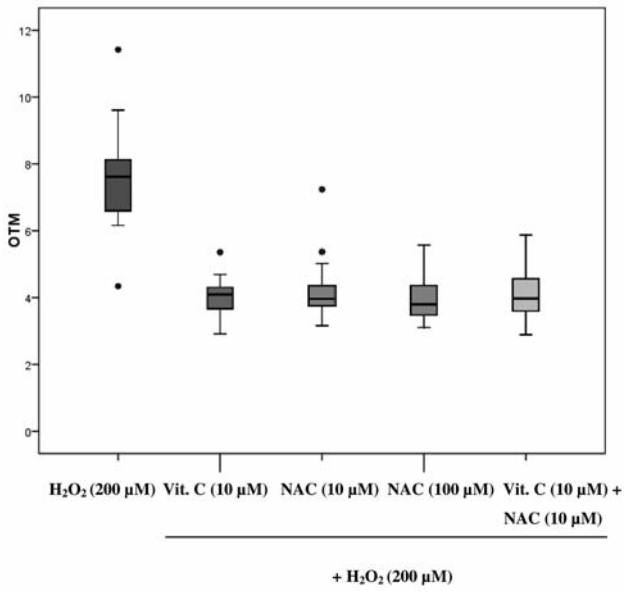

Figure 2. *Boxplot showing the reduction of H_2O_2-induced DNA fragmentation by ascorbic acid (Vit. C), N-acetylcysteine (NAC) and a combination of both (n=20). *Outlier: value more than 1.5 and less than 3 box-lengths from end of box.*

Discussion

The antioxidant protection of human cells includes enzyme-mediated and non-enzymatic defence mechanisms. Superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GSH-px) are the most important antioxidant enzymes. SOD catalyses the reaction of superoxide anion to hydrogen peroxide (H_2O_2); in turn, CAT converts H_2O_2 into water and oxygen. The affinity of CAT for H_2O_2 is relatively low, therefore, some H_2O_2 remains in the cell. GSH-px is capable of detoxifying the remaining H_2O_2 (12). On the other hand, H_2O_2 can also react with redox-active transition metals, such as iron, copper, cadmium and nickel: $(Fe, Cu, Cd, Ni)^{2+} + H_2O_2 \rightarrow (Fe, Cu, Cd, Ni)^{3+} + OH^- + \cdot OH$ (hydroxyl radical).

Hydroxyl radicals are the main source of oxidative damage to the cell. Non-enzymatic antioxidants include vitamins (A, C, E, K), minerals (zinc, selenium), carotenoids (β-carotene), polyphenols (flavonoids, phenolic acids), glutathione, uric acid and co-factors (Q_{10}). Vitamin C and zinc uptake into the cell are mediated by transporter proteins. In epithelial systems, ascorbic acid is transferred into the cell by sodium-dependent solute carrier family member SLC23A1 (13). Zinc is a substrate of transporters of the ZIP family (14). Within the cells, ascorbic acid scavenges ROS, forming more stable ascorbate radicals, thus increasing the protection of DNA and lipids against peroxidative damage and enhancing the repair of oxidized amino acid residues to maintain protein integrity (15). Regarding the respiratory tract, vitamin C was shown to be the major non-enzymatic antioxidant of lining biofilm (16). There are at least two well-established mechanisms by which zinc exerts its antioxidant actions. Firstly, it was shown to protect enzyme sulfhydryls from oxidation, most likely by direct binding to the sulfhydryl group or by inducing a protective conformational change. This is true for many thiol group containing proteins, such as DNA-binding zinc finger (repair) enzymes, but not for all thiol-dependent enzymes. Secondly, zinc antagonizes the formation of hydroxyl radicals, catalyzed by redox-active transition metals in a competitive manner. This is particularly important because transition metals, above all iron and copper, are capable of inducing site-specific radical formation. To remain in solution, iron binds to low-molecular-weight ligands, such as nucleotides, whereas copper is more likely to be complexed with macromolecules such as DNA or proteins. Bound to DNA, the metals can be a source of repetitive radical formation (17).

NAC is widely used to support intracellular glutathione (GSH) synthesis. NAC transport into the cell is yet not fully elucidated, but rapidly deacetylated within the cell, NAC supplies GSH synthesis with L-cysteine [18]. The rate-limiting enzyme during GSH production is γ-glutamylcysteinesynthetase, which catalyses the reaction of L-glutamate and L-cysteine:

L-glutamate + L-cysteine + ATP → L-γ-glumatylcysteine + ADP + P
γ-glutamylcysteinesynthetase

L-γ-glumatylcysteine + glycine + ATP → GSH + ADP + P (19)
glutathione synthetase

Many, if not most, studies investigating DNA protection by micronutrients to date were performed using cell line or animal models. In this context, the use of MOC could be particularly important because of several advantages. Firstly, MOC represent the original target tissue for exogenous oxidative stress in terms of cigarette smoke and other environmental agents. Secondly, compared to single-cell experiments, this model allows repeated treatments without loss of cell material following repetitive incubation and medium renewal (centrifugation, resuspension in medium *etc*.). Thirdly, isolated cells, in contrast to tissue cultures, may have a limited metabolic competence, both for endogenous compounds and xenobiotics, a possibly altered DNA sensitivity and impaired repair capacity (20). Finally, standardized conditions are applied using MOC, which could be somewhat harder to achieve in *in vivo* animals. It is worth mentioning, that after all incubations, MOC were washed twice. Therefore, direct interactions between H_2O_2 and tested substances within the growth medium were excluded as far as possible. Our results clearly show that the tested substances are able to reduce H_2O_2-induced DNA damage in a prophylactic way.

Disclosure

These results were partly presented at the 8th International Conference of Anticancer Research, Kos, Greece.

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