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RESEARCH ARTICLE

Evaluation of the protective effect of ascorbic acid on nitrite- and nitrosamine-induced cytotoxicity and genotoxicity in human hepatoma line

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Abstract
Nitrites are ubiquitous environmental contaminants present in drinking water and foods. Nitrosamines can be formed endogenously from nitrate and nitrite and secondary amines or may be present in food, tobacco smoke, and drinking water. The major goal of this work was to evaluate the cytotoxic, reactive oxygen species (ROS)-producing and genotoxic effects of nitrite and nitrosamines and the possible protection by ascorbic acid in HepG2 cells. It was found that nitrite, N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), and N-nitrosomorpholine (NMOR) decreased cell viability, increased intracellular ROS production, and caused genotoxicity. Compared to untreated cells as determined by alkaline Comet assay, nitrite, NDMA, NDEA, and NMOR raised the tail intensity up to 1.18-, 3.79-, 4.24-, and 4.16-fold, respectively. Ascorbic acid (AA, 10 μM) increased cell viability and reduced ROS production significantly ($p < 0.05$). Additionally, AA treatment decreased the tail intensity caused by nitrite, NDMA, NDEA, and NMOR to 33.74%, 58.6%, 44.32%, and 43.97%, respectively. It can be concluded that ascorbic acid was able to reduce both tail intensity and tail moment in all of the nitrosamine treatments, particularly in NDMA. AA protected HepG2 cells against genotoxic effects caused by nitrosamines. This protection might be through different mechanisms, some of which are not still understood in depth. The future interest will be to understand which pathways are influenced by antioxidants, particularly by AA, and the outcomes of this prevention in other cell line types.

Keywords: *Nitrite; nitrosamine; ascorbic acid; HepG2; genotoxicity; Comet; MTT*

Introduction

Nitrite has been implicated with a variety of long-term adverse effects and has been of interest to public health providers and governmental regulators for the last 40 years. Nitrite and nitrate ions are naturally occurring forms of nitrogen and are present in drinking water and in human diet (green vegetables) (Chung et al. 2003; McMullen et al. 2005). Nitrite is also used as a food preservative against the growth of *Clostridium botulinum* in meat (Tricker and Preussmann 1991; Bruning-Fann and Kaneene 1993a; b; Bartsch and Spiegelhalder 1996; McKnight et al. 1999; Chow and Hong 2002).

Nitrosamines can be formed endogenously from nitrate and nitrite and secondary amines under certain conditions such as strongly acidic pHs of the human stomach (Tricker 1997; Jakszyn and Gonzalez 2006; Bofetta et al. 2008). Humans are exposed to a wide range of NOCs from diet (cured meat products, fried food, smoked preserved foods,

foods subjected to drying, pickled, and salty preserved foods), tobacco smoking, work place, and drinking water (Bartsch and Spiegelhalder 1996; Tricker 1997; Jakszyn and Gonzalez 2006; Bofetta et al. 2008). Three important nitrosamines, namely N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), and N-nitrosomorpholine (NMOR), are classified as probably carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC) (IARC 2000). NDMA is a potent hepatotoxin that can cause fibrosis and tumors in the liver of rats through an activation of CYP450 enzymes (Peto et al. 1991; George et al. 2001; Kasprzyk-Hordern et al. 2005). NDEA, which is present in a wide variety of foods, is a well-known hepatotoxin and induces liver damage at repeated doses in experimental animals (Archer 1989; Jose et al. 1998; Liao et al. 2001; Vitaglione et al. 2004). NDEA is also metabolically activated by CYP450s and it produces reactive

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electrophiles, which increase oxidative stress levels, leading to cytotoxicity, mutagenecity, and carcinogenicity (Archer 1989). Morpholine (1-oxa-4-azacyclohexane) behaves chemically as a secondary amine. Under environmental and physiological conditions, the proven animal carcinogen NMOR is formed by reaction of solutions of nitrite or gaseous nitrogen oxides with dilute solutions of morpholine. Nitrogen oxide (NO) levels may be of importance in nitrosation and in particular pH play a significant role in the formation of NMOR (INCHEM 1996).

The production of reactive oxygen species (ROS) is a well-known physiological process. Several studies have provided evidence that free radical-induced oxidative damage of cell membranes, DNA, and proteins might be the cause of several diseases, such as cancer. Nitrosamines can also be the cause of intracellular ROS production, and antioxidants, such as vitamins, might have a beneficial role in protecting against these diseases (Barnham et al. 2004). Intrinsic antioxidant systems, such as protective enzymatic antioxidants as well as antioxidants available in human diet, provide an extensive array of protection that counteract potentially injurious oxidizing agents (Halliwell and Cross 1994; Ames 1999).

Ascorbic acid (AA, vitamin C) is a -oluble dietary antioxidant that plays an important role in controlling oxidative stress. About 90% of ascorbic acid in the average diet comes from fruits and vegetables (Vallejo et al. 2002). The cytotoxic effects of nitrosamines and protection by AA in several cell lines have been demonstrated before. In a study performed by Robichová and Slamenová (2002), they determined that in CaCo-2 cells at 5.1 mM dose, NMOR caused a 38% decrease in cell viability while AA supplementation (0.5 mmol/L) saved 14% of the cells. Moreover, NMOR (5.1 mM) caused a decrease of 20% in cell viability in V79 cells while AA provided an increase of 18% in viability (Robichová and Slamenová 2002). Moreover, AA was found to be effective in nitrosamine-induced (NDMA, N-nitrosopyrrolidine [NPYR], N-nitrosodibutylamine [NDBA], and N-nitrosopiperidine [NPIP]) DNA damage in HepG2 cells (Arranz et al. 2007; García et al. 2008a; b). One mechanism by which AA exerts its protective effect is that it may reduce the activity of CYP450s, namely CYP2A6 and CYP2E1, that catalyze the metabolic activation of the N-nitrosamines. This reduction might block the production of genotoxic intermediates (Arranz et al. 2007).

The hypothesis that the toxicity nitrite and nitrosamines might be through their oxidative stress-causing effect is still a debate. There is no study in the literature that provides information on the oxidant effects of the nitrite and nitrosamines used in this study and their DNA-damaging effect through such mechanisms. Therefore, the main goal of our study was to show the ROS-producing effect of nitrite and nitrosamines, and whether this effect may be one of the underlying factors of the genotoxicity of these compounds. To our knowledge there is no study in the literature that confirms this relationship. Additionally, the possible protection against ROS production and genotoxicity by AA was also of concern, as AA is an important component of human diet and a powerful reducing agent.

Materials and methods

Chemicals

All chemicals used in the study, including Comet assay chemicals (agarose routine, agarose low melting point, NaCl, Na_2-EDTA, Tris, sodium lauryl sulfate, Triton X-100, Tris-HCl, and ethidium bromide) were purchased from Sigma-Aldrich® (St. Louis, MO) except sodium hydroxide, which was purchased from Carlo Erba® (Rodano, Italy). All chemicals and solvents were of the highest grade available. 5-(and 6-)chloromethyl-2,7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFA) was purchased from Invitrogen® Molecular Probes, Eugene, OR, USA).

Preperation of ascorbic acid, nitrite, and nitrosamines

Stock ascorbic acid solution (1 mM) was prepared in sterile deionized water. The stock was kept divided into portions and aliquots were kept at –80°C in the dark. The aliquots were dissolved immediately before use and diluted to 10 μM with sterile deionized water.

Stock nitrite solution (2 mM) was also prepared in sterile deionized water. The nitrite stock was kept divided into portions and aliquots were kept at –80°C in the dark. The aliquots were dissolved immediately before use and diluted to 20 μM with sterile deionized water.

N-nitrosamines were dissolved in sterile DMSO (0.1%). NDMA, NDEA, and NMOR stocks were prepared at 100 mM, 100 mM, and 30 mM, respectively. The stock solutions were stored in a deep freezer (–80°C).

Cell treatment

The human hepatoma cell line (HepG2) was purchased from American Type Culture Collection (ATCC, HB-8065., Monassas, Virginia, USA). The cells were cultured in Dulbecco's Modified Eagle Medium with 15% fetal calf serum (FCS) and 1% penicillin/streptomycin in culture flasks in 5% CO_2 at 37°C. For sub-cultivation, cells were trypsinized, washed with sterile PBS, and centrifuged at 1500 g for 5 min. Cells only with 15–17 passages were used during the experiments.

MTT assay

Throughout the genotoxicity studies, viability was determined by tryphan blue method using Countess™ Cell Counter (Invitrogen®). No cytotoxicity has been found in AA concentrations between 0.1–10 μM; 10 μM was chosen as it was the highest non-toxic dose to protect the cells from the genotoxic effects of nitrosamines.

HepG2 cells of each group in 200 μL of medium with or without AA (10 μM) in the presence of nitrite, nitroamines (NDMA, NDEA, NMOR) were seeded in eight wells of the 96-well culture plates. After 24 h of culture, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 5 mg/mL) solution (5 mg/mL in Dulbacco's phosphate buffered saline (PBS) with calcium chloride and magnesium chloride) was added. After 2-h incubation at 37°C in 5% CO_2, 200 μL DMSO was added into each well. The dark-blue crystals of MTT-formazan were dissolved by shaking the plates at room

temperature for 2 min and absorbance was then measured on a ELISA reader using Biolise program (Life Sciences International®, Champigny-sur-Marne, France) using wavelength of 562 nm. Each experiment was done in triplicate. Growth rate (%) = A562 $_{treated}$/A562 $_{untreated}$ × 100%.

After cytotoxicity assays, treatment doses of sodium nitrite and nitrosamines were chosen in consideration to the 50% of cell viability (IC$_{50}$ value) and the cells were grouped as follows:

1. Untreated control cells (C);
2. AA-applied cells (AA): Cells were treated with AA (10 μM) for 24 h;
3. Nitrite-treated cells (Nitrite): Cells were treated with nitrite (20 μM) for 30 min;
4. Nitrite-treated and AA applied cells (Nitrite+AA): Cells were treated with AA (10 μM) for 24 h and then treated with nitrite (20 μM) for 30 min;
5. NDMA-treated cells (NDMA): Cells were treated with NDMA (10 mM) for 30 min;
6. NDMA-treated and AA-applied cells (NDMA+AA): Cells were treated with AA (10 μM) for 24 h and then treated with NDMA (10 mM) for 30 min in the presence of AA (10 μM);
7. NDEA-treated cells (NDEA): Cells were treated with NDEA (10 mM) for 30 min;
8. NDEA-treated and AA-applied cells (NDEA+AA): Cells were treated with AA (10 μM) for 24 h and then treated with NDEA (10 mM) for 30 min in the presence of AA (10 μM);
9. NMOR-treated cells (NMOR): Cells were treated with NMOR (3 mM) for 30 min; and
10. NMOR-treated and AA-applied cells (NMOR+AA): Cells were treated with AA (10 μM) for 24 h and then treated with NMOR (3 mM) for 30 min in the presence of AA (10 μM).

ROS production-evaluation of oxidative stress

Seventy-to-80% confluent cells were used in the experiments. The production of intracellular ROS was measured as described earlier by Loikkanen et al. (1998). All the study was conducted in the dark. ROS was detected by CM-H$_2$DCFA using a microplate reader. For the assay, cells were plated in 24-well multiwells at a number of 2×10^5 cells per well with or without AA (10 μM) for 24 h. Later culture media was removed and nitrite or nitrosamine including culture media was put in the wells for 30 min. Later the cells were loaded with 5 μM CM-H$_2$DCFA in 0.5 ml of PBS buffer for 30 min at room temperature. The cellular esterase activity results in the formation of the non-fluorescent compound 2′,7′-dichlorofluorescin (DCFH). DCFH is rapidly oxidized in the presence of ROS to a highly fluorescent 2′,7′-dichlorofluorescein (DCF). DCF fluorescence was measured at time points of 0, 30, 60, and 90 min with a PerkinElmer Victor 3 1420 multiwell fluorometer (Perkin

Elmer®, Buckinghamshire, UK) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, and Wallac 1420 Manager Program was used. Background fluorescence was obtained from cell-free wells containing 5 μM DCF in 0.5 ml of PBS and subtracted from the fluorescence values found. The multiwell plate was kept in a cell culture incubator between the measurements. The exposures were repeated 3–4-times with three parallel measurements. Fluorescence values were normalized to the cell numbers. For each condition, 8-wells were used and the mean was given as a result. This parameter gives a very good evaluation of the degree of cellular oxidative stress.

As shown in Figure 1, nitrite and all the nitrosamines used in the study raised the intracellular ROS levels. AA was able to reduce ROS production significantly ($p < 0.05$) when compared to nitrite and nitrosamine treatments alone.

Comet assay

The alkaline comet assay was performed according to Hininger et al. (2002) with minor modifications. Assay was performed on two different days on triplicate slides and the median of two days and six slides was given as the result. Briefly, slides were coated with agarose (1% in PBS) the day before the experiment was performed. Agarose low melting point was prepared in PBS as 0.6% and kept at 37°C before the cells were added. Cells were prepared as 15,000 cells/10 μl and 50 μl of the cell suspension was added to 450 μl of agarose low melting point, and 100 μl of the mixture was put on each slide and a cover slip was put on each slide. Slides were left on ice in order to allow agarose to solidify. After gently removing the cover strip, the cells were lysed with cold lysing solution (2.5 M NaCl, 0.5 M Na$_2$-EDTA, 10 mM Tris, 1% sodium lauryl sulfate,1% Triton X-100, 10% DMSO, pH 10) to enable DNA unfolding. After 1 h in the dark at 4°C, the slides were washed three times with 'Wash buffer' (0.4 M Tris-HCl, pH 7.4) and immersed in freshly prepared 'Electrophoresis buffer' (1 mM Na$_2$-EDTA and 300 mM NaOH, pH 13) for 30 min in order to allow the unwinding of the DNA, and then electrophoresis was performed at 25 V/300 mA for 30 min. After electrophoresis, slides were neutralized in 'Wash buffer' again and stained with ethidium bromide (20 μg/ml, 50 μl/ slide), covered with a cover strip prior to analysis. For quantification, a fluorescence microscope (Carl Zeiss®, Germany) connected to a charge-coupled device (CDC) was used. A computer-based analysis system (Comet IV) was used to determine the extent of DNA damage after electrophoresis migration of DNA fragments in the agarose gel. For each condition 50 randomly selected comets on each slide were scored and Tail DNA (%) (the average of the percentage of DNA in the tail) and the tail moment (the distance between the centre position of the head and the centre gravity to the tail, arbitrary units) were determined using three slides prepared as described above.

Statistical analysis

All of the results were expressed as mean ± standard error of mean (SEM). The differences among the groups were evaluated with Student's *t*-test. The mean difference was considered significant at the 0.05 level.

Results

Results of MTT assay for nitrite and nitrosamines are shown in Figure 2. It was found that AA protected the cells against nitrite and nitrosamines. AA supplementation in the HepG2 cells treated with nitrite, NDMA, NDEA, or NMOR caused enhanced cell viabilities, and this protection was found to be statistically significant at IC50 value in nitrite- and nitrosamine-treated cells (all, $p < 0.05$).

As shown in Figure 1, both nitrite and nitrosamines enhanced ROS production in 90 min of exposure, and ROS production was found to be significantly lower at 30, 60 and 90 min AA-treated cells (all, $p < 0.05$).

The comet assay results are given in Figures 3 and 4. When compared to untreated cells, nitrite ($p > 0.05$), NDMA ($p < 0.05$), NDEA ($p < 0.05$), and NMOR ($p < 0.05$) raised the tail intensity up to 1.18-, 3.79-, 4.24-, and 4.16-fold, respectively. AA was able to reduce the tail intensity caused by nitrite, NDMA, NDEA, and NMOR to 33.74%, 58.6%, 44.32%, and 43.97%, respectively, and

these reductions were statistically significant when compared to each individual toxic compound applied group (all, $p < 0.05$). Besides, nitrite, NDMA, NDEA, and NMOR increased the tail moment up to 1.94, 6.04, 6.05, and 5.70, respectively. AA (10 μM) enabled a reduction of 26.81%, 30.00%, 22.60%, and 21.85% in the tail moment in nitrite, NDMA, NDEA, and NMOR-treated cells, respectively, and these reductions were statistically significant when compared to each individual toxic compound applied group (all, $p < 0.05$).

DMSO (0.1%) was also tested for DNA damage by Comet assay; however it did not induce any damage. Tail intensity (%) was 3.45 and tail moment (in arbitrary units) was 1.40.

Discussion

Epidemiological and clinical studies have shown in the past decade that ROS induce oxidative damage of cell membranes, DNA, and proteins, and might be the cause of aging
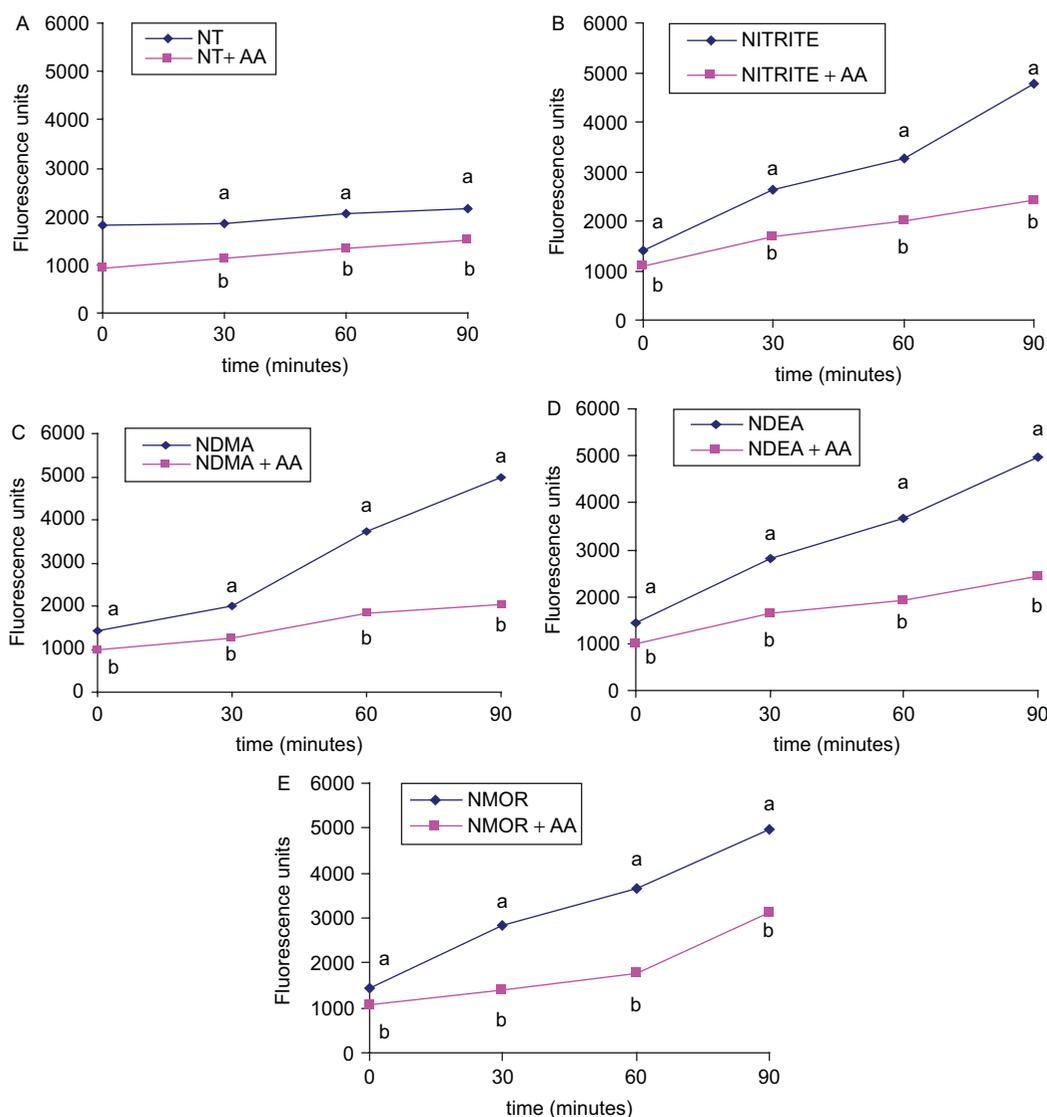

Figure 1. Effects of nitrite and nitrosamines on ROS production and evaluation of protection by ascorbic acid at 30, 60, and 90 min. Ascorbic Acid (AA, 10 μM), nitrite (20 μM), NDMA (N-nitrosodimethylamine, 10 mM); NDEA (N-nitrosodiethylamine; 10 mM), NMOR (N-nitrosomorpholine, 10 mM). [a, b] Superscripts of different letters differ significantly ($p < 0.05$) from each other.
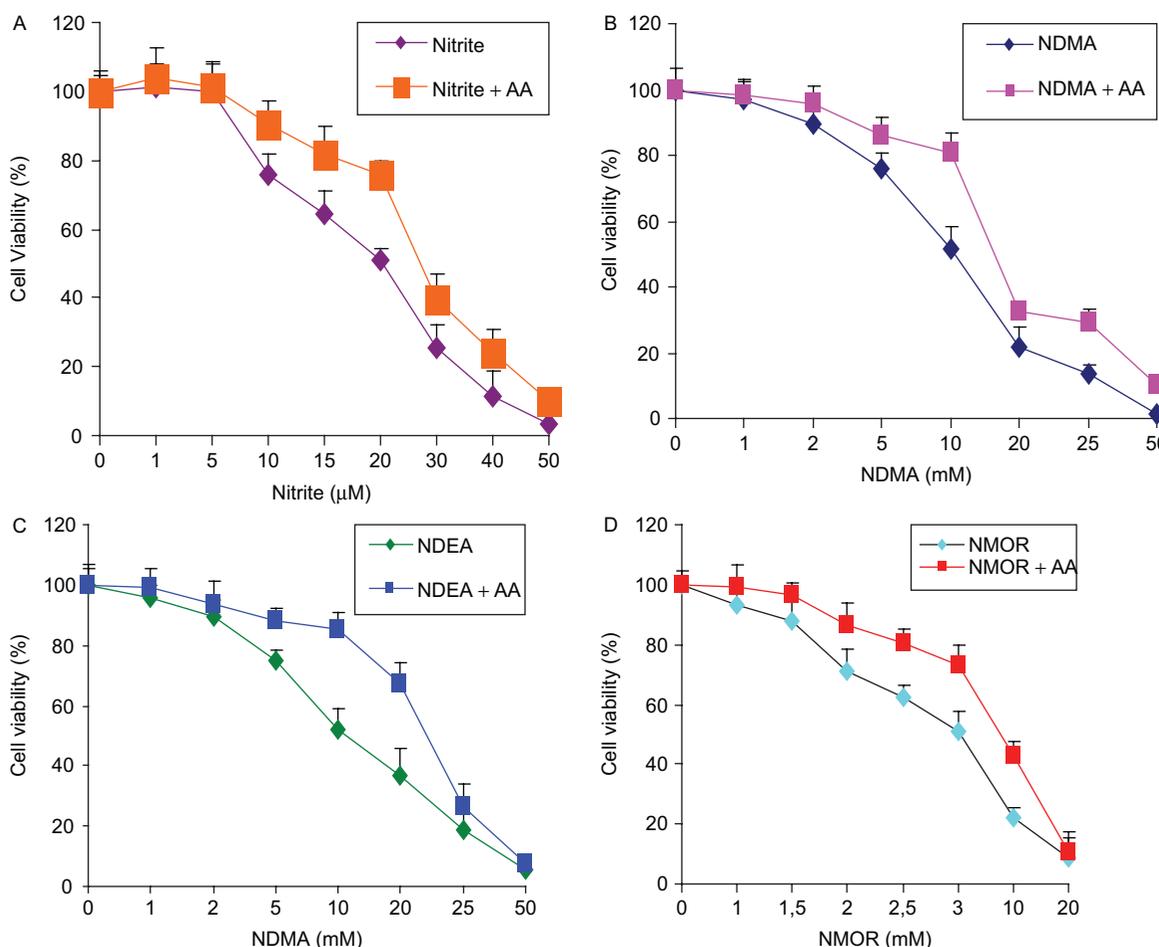
Figure 2. Effects of nitrite at various µM concentrations and nitrosamines at various mM concentrations on cell viability and evaluation of prevention of cell death by ascorbic acid at 10 µM by using MTT assay. Ascorbic Acid (AA, 10 µM), NDMA (N-nitrosodimethylamine); NDEA (N-nitrosodiethylamine), NMOR (N-nitrosomorpholine).

and diseases like cancer (Athar 2002; Valko et al. 2004). Endogenous DNA lesions are genotoxic and induce mutations (Platz et al. 2000; Willett 2001).

The effect of ROS is balanced by the antioxidant action of non-enzymatic antioxidants as well as antioxidant enzymes. Epidemiological data suggest that cancer risk may be reduced by simple daily diet changes (Platz et al. 2000; Willett 2001). Non-enzymatic antioxidants involve AA and several other vitamins and trace elements like selenium. About 90% of AA in the diet comes from fruits (especially strawberry, orange, lemon) and vegetables (broccoli, cauliflower, tomato, etc.) (Castenmiller et al. 1999; Valko et al. 2004).

Cancer is a leading cause of death worldwide, and diet is thought to play a substantial role in its etiology. The detrimental effects of different foods, food components, and contaminants in food have been widely studied in the laboratory animals, and several epidemiologic studies were conducted (Abnet 2007). Particularly, nitrites and nitrosamines are of concern because several animal studies have confirmed harmful effects (Chung et al. 2003; McMullen et al. 2005). NDMA, NDEA, and NMOR belong to the group of carcinogenic nitrosamines (IARC 2000; George et al. 2001). The protective effects of several compounds such as organosulfurs and isothiocyanates, present in vegetables towards N-nitrosamine

induced oxidative damage have been tested before (Arranz et al. 2006; 2007). The protective role of AA and several other vitamins against oxidative stress induced by environmental mutagens including NDMA, N-nitrosopyrolidine (NPYR), N-nitrosopiperidine (NPIP), NDEA, and NMOR has been demonstrated by several other studies (Bast et al. 1996; Claycombe and Meydani 2001; Halliwell 2001; Garcia et al. 2008a and 2008b). NMOR has been shown to exert cytotoxic and genotoxic properties in Cac2 and V79 cells, even in the absence of S9 fraction and cofactors. In the other studies performed by Robichová et al. (2004a; b), it was shown that NMOR interacted with DNA in HepG2, V79, and VH10 cells. However, AA protection against NMOR-induced genotoxicity could only be shown by Comet assay. No protection was seen in chromosomal aberrations (Robichová et al. 2004a).

Both nitrite and nitrosamines raised the intracellular ROS levels in 90 min. The reason behind the genotoxicity of these compounds might be the ROS-producing effect. Moreover, the inhibition of nitrosamine formation by AA by reacting with nitrite/nitrosating agents faster than secondary amines might be another explanation (Gichner and Velemínský 1988; Tanaka et al. 1998; Schorah 1999). Furthermore, such antioxidants may inhibit the genotoxicity/mutagenicity of nitrosamines by activating the DNA repair (Gichner and Velemínský 1988).
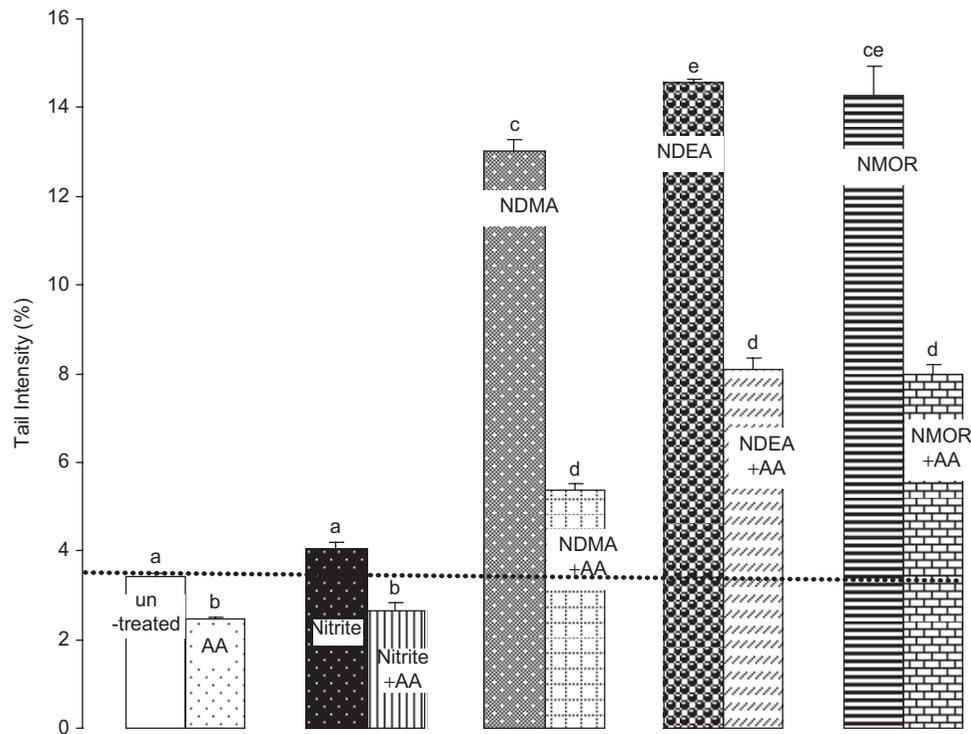
Figure 3. Evaluation of tail intensities obtained from comet assay by nitrite and nitrosamines and protection by ascorbic acid. Ascorbic acid (AA, 10 μM), Nitrite (20 μM), NDMA (N-nitrosodimethylamine, 10 mM); NDEA (N-nitrosodiethylamine; 10 mM), NMOR (N-nitrosomorpholine, 10 mM). [a, b, c, d, e, f] Superscripts of different letters differ significantly ($p < 0.05$) from each other.
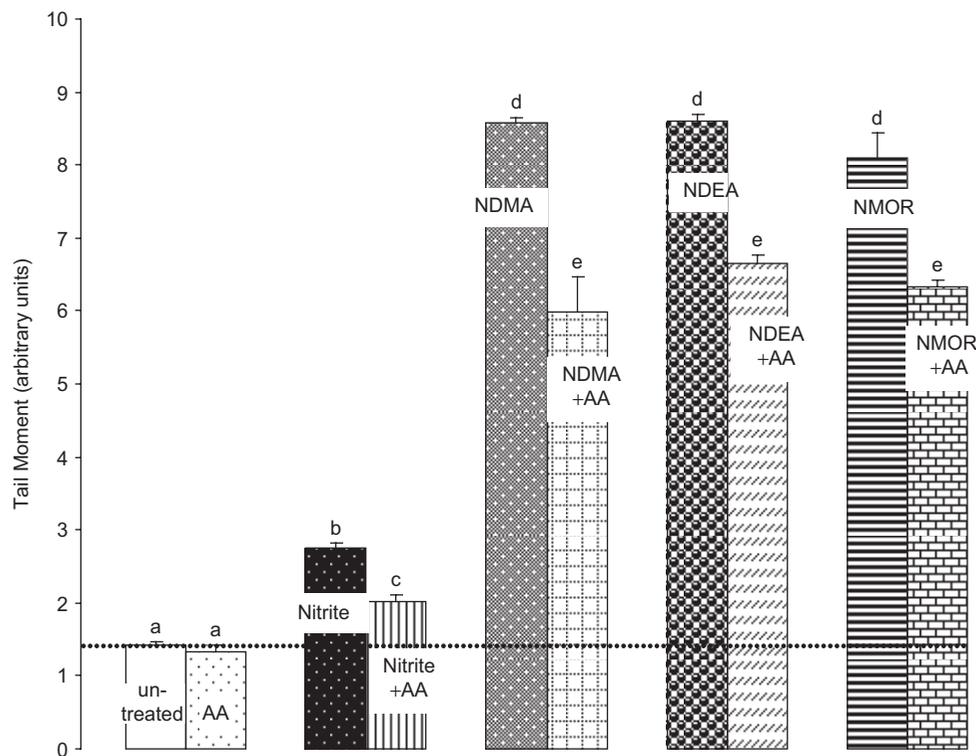

Figure 4. Evaluation of tail moments obtained from comet assay by nitrite and nitrosamines and protection by ascorbic acid. Ascorbic acid (AA, 10 μM), nitrite (20 μM), NDMA (N-nitrosodimethylamine, 10 mM); NDEA (N-nitrosodiethylamine; 10 mM), NMOR (N-nitrosomorpholine, 10 mM). [a, b, c, d, e] Superscripts of different letters differ significantly ($p < 0.05$) from each other.

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Here we confirm the DNA damaging effect of nitrosamines as shown in other studies (Robichová et al. 2004b; Arranz et al. 2006; 2007; Garcia et al. 2008a; b). Additionally, we used sodium nitrite to show the genotoxic effects of nitrite alone. AA supplementation was capable of reducing both tail intensity and tail moment in all of the nitrosamine treatments, particularly in NDMA. This may be related to AA's reduction of CYP2E1 and CYP2A6. CYP2E1 is responsible for α-hydroxylation of N-alkylnitrosamines with short alkyl chain, whereas cyclic nitrosamines like NPYR, NPIP, and NMOR may be activated by CYP2A6 and by CYP2E1 to a lesser extent (Kamataki et al. 2002). Furthermore, inhibition of CYP450 enzymes may not be the only mechanism underlying the protection of AA. Alternative mechanisms by AA may be as follows: ROS scavenging capacity, the conversion of reactive compounds to less toxic and easily excreted compounds, alteration of cell proliferation, stimulation of DNA-repair induced by nitrosamines, induction of Phase II enzymes, and NAD(P):quinine oxidoreductase activity (Roomi et al. 1998; Chaudière and Ferrari-Iliou 1999; Gamet-Payrastre et al. 2000; Surh et al. 2001; Surh 2002).

We chose Comet assay as it is a simple, visual, and quantitative technique for detecting DNA strand breaks. It has gained popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring, and genotoxicity testing. Evaluation of DNA damage by Comet assay gives accurate and reproducible results. HepG2 cells were used in our study as these cells exert the same properties of human hepatocytes other than levels of phase I enzymes (Appel and Graf 1982). This may be an important factor in the metabolic activation as these nitrosamines may also need metabolic activation to show their toxic effects including their carcinogenesis. The nitrosamines are cleaved by one-electron transfer from the hemoprotein iron of cytochrome P450, resulting in NO and secondary amine according to Appel and Graf (1982). Further hydroxylation of the secondary amines causes the formation of DNA-reactive N-hydroxylamines in the presence of 'nitroreductase' enzyme (Wang and Higuchi 1995). Therefore, these nitrosamines may be even more toxic to primary hepatocytes, as these hepatocytes are able to exert metabolic activation more than HepG2 cells.

To summarize, AA protects HepG2 cells against cytotoxic and genotoxic effects caused by nitrosamines. Dietary antioxidants can be a savior when exposure to dietary genotoxic/carcinogenic compounds is the case. The future interest is to understand which pathways are being influenced by antioxidants in nitrite and nitrosamine toxicity, particularly by AA and the outcomes of the protection in other cell line types.

Declaration of interest

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