Investigation of the role of extracellular H₂O₂ and transition metal ions in the genotoxic
action of ascorbic acid in cell culture models

Tiago L. Duarte^{*}, Gabriela M. Almeida, George D. D. Jones

Radiation and Oxidative Stress Group, Department of Cancer Studies and Molecular
Medicine, Biocentre, University of Leicester, Leicester, LE1 7RH, U.K.

^{*}Corresponding author. Tel.: +44-116 2525842. *E-mail address*: td13@le.ac.uk (T. L. Duarte)

Abstract

In the presence of oxygen, ascorbic acid (AA) is unstable in aqueous media and oxidises to dehydroascorbate (DHA), generating reactive intermediates such as ascorbate free radical and H₂O₂. It is proposed that the cytotoxicity of AA is due to the extracellular production of H₂O₂ and that this is mediated by transition metal ions present in cell media. Here we investigate the role of extracellular H₂O₂ and metal ions in the genotoxicity of AA in cell culture models. Our preliminary results confirmed that physiological concentrations of AA were not toxic to confluent human fibroblasts, although they inhibited the proliferation of cells at low density. No inhibition was observed with ascorbic acid 2-phosphate (AA2P), a vitamin C derivative that remains stable in culture media. Furthermore, high concentrations of AA induced DNA strand breakage in a dose-dependent manner, whereas DHA and AA2P were not genotoxic. The genotoxic effect of AA was transient, required the formation of extracellular H₂O₂ and the presence of intracellular iron, but not of extracellular transition metal ions. These observations further clarify the pro-oxidant effect of AA solutions in cell culture models. The possibility that intravenous administration of high-dose AA may cause a similar genotoxic effect *in vivo* is discussed.

Keywords: Ascorbic acid; DNA damage; H₂O₂; Transition metal ions; Genotoxicity

1. Introduction

Vitamin C (ascorbic acid, AA) is an important antioxidant in human plasma. Ascorbate, the mono-anion form of AA and the predominant species at physiological pH, is an efficient electron donor in many biological redox reactions, capable of replacing potentially damaging, highly oxidising radicals by the poorly reactive ascorbate free radical (AFR). Furthermore, ascorbate can replenish other important antioxidant molecules (reviewed by May, 1999; Duarte and Lunec, 2005). However, AA has paradoxical effects on the viability of cell cultures. Whilst some studies showed that AA stimulates collagen synthesis (Blanck and Peterkofsky, 1975; Freiburger et al., 1980; Murad et al., 1981) and proliferation in human diploid fibroblasts (HDFs) (Hata et al., 1988; Chan et al., 1990; Chepda et al., 2001), other studies reported a cytotoxic effect (Peterkofsky and Prather, 1977; Michiels et al., 1990; Peterszegi et al., 2002). The cytotoxicity of AA has been associated with the formation of H_2O_2 in the extracellular environment (Peterkofsky and Prather, 1977; Galloway and Painter, 1979; Prasad et al., 1979; Peterszegi et al., 2002). The formation of H_2O_2 is thought to result from the aerobic 'auto-oxidation' of ascorbate in the culture medium at physiological temperature and pH (Winkler, 1987). The observation that the oxidation of ascorbate at neutral pH is accelerated by copper or iron and slowed down in the presence of chelating agents prompted the proposition that the oxidation of AA depends on the presence of trace levels of catalytic transition metals in most solutions employed in laboratory research (Buettner, 1988). Recently, it was proposed that intravenous administration of AA can be used as a way of delivering H_2O_2 to tissues, to selectively kill cancer cells, which are thought to have lower levels of endogenous antioxidant defences (Chen et al., 2005). However, transition metal ions are not usually available in human plasma (Miller et al., 1990), so it would be important to understand if the formation of H_2O_2 is transition metal ion-dependent.

Whilst H_2O_2 itself is a poorly reactive molecule, in the presence of transition metal ions it can be converted to the highly reactive hydroxyl radical (Halliwell and Gutteridge, 1999). The production of H_2O_2 in cell culture media is thus expected to damage cellular DNA. The genotoxic effects of AA on cell culture models have been recently assessed using the alkaline comet assay, a sensitive microelectrophoretic

technique for the direct detection of several types of DNA damage in single cells, including DNA strand breaks, alkali-labile sites and incomplete excision repair sites (Singh et al., 1988). Results were conflicting, with AA causing DNA damage in some studies (e.g. Anderson et al., 1994; Singh, 1997) but not in others (e.g. Szeto and Benzie, 2002). Despite the inconsistent results, AA is commonly employed as a supposed antioxidant in order to counteract the genotoxic action of numerous agents (e.g. Re et al., 1997; Blasiak et al., 2000, 2003; Wozniak and Blasiak, 2003, 2004; Blasiak and Kowalik, 2001; Robichova and Slamenova, 2002).

In this study, we investigated the pro-oxidant effect of vitamin C solutions in cell culture models. In particular, our aim was to clarify the mechanisms leading to the formation of DNA damage in HDFs, by assessing the involvement of extracellular H₂O₂ and transition metal ions, and to relate these with cell viability endpoints. To circumvent the problems associated with AA auto-oxidation, we used ascorbic acid 2-phosphate (AA2P), a vitamin C derivative that remains stable in cell culture media at 37 °C (Hata and Senoo, 1989). AA2P is hydrolysed by cellular phosphatases to AA, leading to an accumulation of intracellular ascorbate (Furumoto et al., 1998; Savini et al., 1999). The current study helps addressing the contradictory findings of studies of the toxic effects of AA on cell culture models and the possible physiological relevance of the often-cited pro-oxidant effect of AA solutions.

2. Materials and Methods

2.1. Reagents

Minimal essential medium (MEM) with Earle's salts and non-essential amino acids, foetal bovine serum (FBS) and Glutamax-I were purchased from Invitrogen (Paisley, UK). Magnesium L-ascorbate 2-phosphate (AA2P) was obtained from Wako Pure Chemical Industries (Neuss, Germany). Superoxide dismutase (SOD) from bovine liver was obtained from Merck (Darmstadt, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.2. Cell lines and culture conditions

GM5399 primary HDFs established from skin biopsy of an apparently healthy donor were purchased from the NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, New Jersey, USA) and grown as a monolayer culture at 37 °C, in a humidified atmosphere containing 5 % CO₂. HDFs were grown in MEM with Earle's salts supplemented with 2 mM Glutamax-I and 10 % (v/v) FBS, and routinely passaged by trypsinisation when nearly confluent. All experiments were performed at passage numbers 10-17 and cells were confirmed to be free of mycoplasma contamination. All solutions were freshly prepared and sterilised by passing through a 0.22 µm membrane immediately prior to the experiments. For DNA damage experiments, vitamin C solutions were prepared in low-serum medium (0.5 % FBS) and incubated at 37 °C in the dark for a certain period of time. Confluent HDFs were then incubated with these solutions for 30 minutes on ice, in the dark.

2.3. Single-cell gel electrophoresis (comet assay)

DNA damage was measured using the alkaline comet assay described by Singh et al. (1988) with modifications. HDFs were harvested by trypsinisation and suspended in 0.6 % low melting point agarose. Eighty microliters of the agarose gel (containing approximately 3×10^4 cells) were dispensed onto glass microscope slides previously coated with 1% normal melting point agarose. The agarose was allowed to set on ice under a coverslip and the slides left overnight in ice-cold lysis buffer (100 mM disodium EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10 containing 1 % triton X-100 v/v added fresh). Slides were washed with distilled water and then placed in a horizontal electrophoresis tank containing ice-cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM disodium EDTA, pH ≥ 13) for 20 minutes and electrophoresed at 30 V (0.7 V/cm) and 300 mA for 20 minutes. Slides were neutralised with 0.4 M Tris-HCl, pH 7.5 for 20 minutes and washed with double-distilled water, then allowed to dry. All procedures were carried out under subdued light to minimise background DNA damage. For staining, slides were re-hydrated in distilled water, incubated with a freshly made solution of 2.5 µg/mL propidium iodide for 20 minutes, washed again for 30 minutes and allowed to dry. Comets were visualised in the fluorescence microscope at a magnification

of 200×. Images were captured by an on-line CCD camera and analysed with the Komet Analysis software version 5.5 (Andor Bioimaging, Nottingham, UK). A total of 100 cells were analysed per sample, 50 per duplicate slide. The percentage of DNA in the tail of the comet (% tail DNA) was calculated for each cell by the Komet Analysis software.

2.4. Cell viability assays

Cell viability was routinely assessed by the trypan blue dye exclusion assay. In addition, cell viability was determined by the colorimetric MTT assay (Mosmann 1983). HDFs in 96-well plates were incubated with 50 µl of 0.5 mg/ml MTT at 37 °C for 4 hours. The MTT solution was then removed and the coloured formazan crystals formed by the cells were solubilised in 100 µl of DMSO. Plates were gently shaken for 5 minutes and the absorbance of the formazan product read at 544 nm in a FLUOstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany). For proliferation studies, HDFs were seeded at a low density (0.33×10^4 cells/cm²) and left to adhere for approximately 24 hours. The culture medium was then replaced with 20, 100 or 500 µM solutions of either AA or AA2P dissolved in complete growth medium. Control cells were incubated with medium alone. The medium was changed daily and 0, 3, 5 or 7 days later cells were harvested by trypsinisation and counted using the CASY Cell Counter and Analyser System (Schärfe System GmbH, Germany).

2.5. Measurement of AA concentration

AA was added to low-serum medium to a final concentration of 100 µM. At regular intervals, a 0.5 ml aliquot was collected and added to 4.5 ml of ice-cold 10 % metaphosphoric acid. The solution was centrifuged at $1260 \times g$ for 10 minutes at 4 °C to remove any debris and the supernatant kept at -70 °C until ready to analyse. AA was measured by high-performance liquid chromatography (HPLC) with UV detection (254 nm). The HPLC included an isocratic LC pump 250 (Perkin Elmer, Norwalk, CT, USA), an auto-sampler model 542 (ESA) and a UV detector UVD340S (Dionex, Camberley, UK). AA was separated on a Luna 5u C18 (2) HPLC column (150 × 4.6 mm) (Phenomenex, Macclesfield, UK) with an injection volume of 50 µl. The mobile phase consisted of 15 mM phosphate buffer, pH 6. The flow rate was 0.4 ml per minute. The

retention time of AA was approximately 5 minutes. AA was quantified using a standard curve.

3. Results

The viability of confluent HDFs incubated with a physiological concentration (100 μ M) of AA or AA2P for 24 hours was determined with the MTT assay. As depicted in Fig. 1A, none of the vitamin C supplements reduced the relative viable cell number when compared with control cells. Likewise, cell viability was > 95 % in all three groups, as judged by the ability to exclude trypan blue (data not shown). So, it can be concluded that AA or AA2P did not have an effect on the viability of confluent HDFs. For cell proliferation studies, HDFs were seeded at a low density (0.33×10^4 cells/cm²) and incubated with 20, 100 or 500 μ M solutions of either AA or AAP or growth medium alone for up to 7 days. Medium was changed daily. In preliminary experiments, we observed that control HDFs divided exponentially for 5 days and started to plateau at around the seventh day in culture (data not shown). HDFs incubated with the two vitamin C derivatives were therefore harvested and counted at day 5. Notably, AA, but not AA2P, caused a dose-dependent reduction in the proliferation of HDFs seeded at low cell density (Fig. 1B). The highest concentration of AA caused cell death within a few hours, as indicated by cell shrinkage and membrane blebbing, followed by detachment (data not shown).

The oxidation of AA in culture medium is well documented in the literature (e.g. Chepda et al., 2001) and was confirmed here. Results depicted in Fig. 2 show that AA was lost from culture medium in a time-dependent manner. AA loss was faster during the first 2 hours of incubation.

For DNA damage studies, confluent HDFs were exposed to increasing concentrations of H₂O₂ or AA on ice to prevent any DNA repair. All treatments were carried out in medium with a minimal amount of serum (0.5 %) in order to avoid interference of the serum constituents (e.g. transition metal ions). H₂O₂ produced the anticipated dose-response relationship in the comet assay (Fig. 3A, top). Vitamin C solutions were prepared immediately before use and incubated at 37 °C for 1 hour in the dark to allow for auto-oxidation. Only then were the solutions added to cells and

incubated on ice for 30 minutes, before processing them for the comet assay. AA caused DNA damage in a dose-dependent manner (Fig. 3A, bottom). An increase in DNA damage was detected with AA concentrations equal to or higher than 100 μ M. In contrast, the oxidised form of vitamin C, DHA, and the stable AA derivative, AA2P, were not able to cause DNA damage at concentrations up to 1 mM (Fig. 3B). Although some AA may be released from AA2P as a consequence of the activity of serum phosphatases (Chepda et al., 2001), no DNA damage was observed here when AA2P was pre-incubated in complete growth medium (10 % FBS) (data not shown), which indicates that the contribution of serum phosphatases was insignificant. Overall, these experiments suggest that DNA damaging species are produced in the medium during the pre-incubation at 37 °C when the free, reduced form of AA is present. To test this hypothesis, AA solutions (500 μ M) were pre-incubated at 37 °C for different periods of time before adding to cells (Fig. 3C). Notably, no DNA damage was detected when AA solutions were prepared and immediately added to cells on ice. However, DNA damage levels were significantly elevated when AA solutions were pre-incubated for 1 or 2 hours at 37 °C. These results suggest that it is a product formed during AA auto-oxidation, rather than AA itself, that is responsible for DNA damage. Longer pre-incubation times (4-8 hours) decreased AA-induced DNA damage substantially, which indicates that the species responsible for DNA damage are only transiently produced and are not stable in solution for long.

Previous reports have shown that AA auto-oxidation in culture medium in aerobic conditions leads to the formation of H₂O₂ (Clement et al., 2001; Wee et al., 2003). To test the hypothesis that the genotoxicity of AA is due to the formation of H₂O₂ in the culture medium, experiments were performed in which AA solutions were pre-incubated at 37 °C in the presence or absence of the specific H₂O₂-degrading enzyme catalase (CAT; EC Number: 1.11.1.6). As depicted in Fig. 4, CAT completely abolished AA-induced DNA damage. As the solutions were added to cells on ice, the enzyme would have only been active during the pre-incubation time. Therefore, it can be concluded that the formation of H₂O₂ during the pre-incubation at 37 °C was essential for the ability of AA to damage the DNA. The addition of superoxide dismutase (200 U/ml; EC Number: 1.15.1.1) during

pre-incubation did not abolish the DNA damage elicited by AA solutions (data not shown).

The oxidation of AA in solution is thought to be dependent on the presence of trace levels of transition metal ions (Buettner, 1988). The contribution of extracellular transition metal ions to AA genotoxicity was therefore investigated by pre-incubating AA at 37 °C in the presence of equimolar amounts of different metal chelators (Fig. 4). Addition of exogenous trivalent (diethylenetriaminepentaacetate, DTPA) and divalent (ferrozine, FZN) metal ion chelators did not seem to affect the AA-induced DNA damage levels, whereas two iron-specific chelating agents, apo-transferrin (apo-Tf) and desferrioxamine (DFO), augmented the damage. On the other hand, co-incubation with 2,2'-bipyridyl (BIP) prevented DNA strand breakage completely. However, this was probably due to the ability of this metal chelator to permeate cells quickly and hence chelate intracellular reactive iron during the incubation with AA on ice. In fact, AA-induced DNA damage was also completely abrogated when cells were pre-incubated with 300 µM DFO for 14 hours, which allows for efficient intracellular accumulation of the chelator (Halliwell, 1989). Taken together, these results show that, whilst the DNA damage elicited by AA solutions is suppressed by effective chelation of intracellular iron, the formation of the damaging species (i.e. H₂O₂) in the medium during AA auto-oxidation is not prevented by extracellular chelating agents.

4. Discussion

The effects of AA on cell proliferation are controversial. However, careful analysis of the literature reveals that the studies reporting that AA stimulates the proliferation of cultured HDFs were generally performed with confluent cells (Hata et al., 1988; Chan et al., 1990; Chepda et al., 2001), whereas the studies showing a cytotoxic effect used cells at low density and/or high (millimolar) concentrations of AA (Peterkofsky and Prather, 1977; Michiels et al., 1990; Peterszegi et al., 2002). The current study confirms that physiological concentrations of AA are not toxic to confluent HDFs, although they inhibit the proliferation of cells treated at low density in a dose-dependent manner. We also confirm that the stable vitamin C derivative, AA2P, is not cytotoxic, even if supplied at a high concentration (500 µM). Our results thus agree with previous

evidence that the cytotoxic activity of AA is exclusive of those vitamin C derivatives that auto-oxidise in culture medium (e.g. L-AA and sodium-L-ascorbate), whereas stable AA derivatives (e.g. L-AA2P magnesium salt and L-ascorbic acid 2-sulfate) are not toxic (reviewed by Sakagami and Satoh, 1997).

To date, the studies that have addressed the genotoxicity of AA by employing the alkaline comet assay have produced conflicting results. Whilst *ex vivo* exposure of human lymphocytes to AA concentrations of up to 200 μ M failed to induce any DNA damage in one study (Szeto and Benzie, 2002), a dose-dependent increase in DNA damage was observed in another study between 200-5000 μ M (Anderson et al., 1994). Furthermore, sodium ascorbate (25-100 μ M) caused DNA strand breakage in HDFs and lymphocytes (Singh, 1997). Our study showed that AA causes DNA damage in a dose-dependent manner at concentrations ≥ 100 μ M. Higher AA concentrations were required in our study, which is probably due to the fact that Singh (1997) treated HDFs at low density and we worked with confluent monolayers. In fact, H₂O₂-induced DNA damage is reduced in confluent cell cultures (Duthie and Collins 1997).

It is worth noting that several studies have recently employed AA as a supposed antioxidant in order to clarify the genotoxic action of numerous agents (e.g. Re et al., 1997; Blasiak et al., 2000, 2003; Wozniak and Blasiak, 2003, 2004; Blasiak and Kowalik, 2001; Robichova and Slamenova, 2002). Typically, cells were pre-incubated with AA for a short period of time (less than an hour) before exposure to the test agent or co-incubated with the test agent for a similar period of time. At least in some cases, vitamin C *per se* appeared to increase DNA damage (Blasiak et al., 2000, 2003). On the basis of our findings, the use of AA in genotoxicity tests needs to be considered with caution.

To clarify their genotoxic activity in cell culture models, we have incubated vitamin C solutions in aerobic conditions at 37 °C for different periods of time before adding them to cells. HDFs were incubated with these solutions at 0 °C, which suppresses AA accumulation (Butler et al., 1991). Consequently, any genotoxic effects could be attributed to a product of AA auto-oxidation capable of entering cells by passive diffusion, rather than to AA itself. Using this different approach, we could show that only when vitamin C solutions were incubated at 37 °C to allow for auto-oxidation before adding to cells, AA, but not the oxidised form of the vitamin (DHA) or a stable derivative

(AA2P), was able to damage the DNA. The effect was maximal within the first 2 hours of AA incubation and diminished at longer incubation times. Taken together, our results show that DNA damage is not caused by AA itself but rather by products of its oxidation formed during the first two hours of incubation. Experiments where CAT was present during the pre-incubation period confirmed extracellular H₂O₂ as the mediator of DNA damage formation. Regression analysis of the dose-response curves of DNA damage formation induced by AA or H₂O₂ indicates that, under the experimental conditions employed herein, 1 mM AA induced an amount of DNA damage equivalent to that induced by 56 μ M H₂O₂. This is in good agreement with the study of Wee et al. (2003), who observed that approximately 60 μ M of H₂O₂ were generated from 1 mM AA within one hour of incubation in cell medium at 37 °C. H₂O₂ is poorly reactive and it can easily reach the nuclear compartment where, in the presence of intracellular reactive iron, it can yield highly reactive oxygen species that damage the DNA (Halliwell and Gutteridge, 1999). The formation of extracellular H₂O₂ may explain the loss of cell viability observed at low cell density, especially following exposure to high (millimolar) concentrations of AA. However, low concentrations of H₂O₂ are reasonably well tolerated by cells, so we would expect the pro-oxidant effect to be minimal when AA is supplied to confluent cells at physiologic concentrations (i.e. $\leq 100 \mu$ M). AA2P, on the other hand, did not have a pro-oxidant effect and did not affect cell viability, even when HDFs were treated at a low cell density. Therefore, studies of the intracellular effects of vitamin C that require the use of cells at low density would benefit from the use of a stable vitamin C derivative like AA2P.

It has recently been proposed that high (grams) intravenous concentrations of AA may be a clinically useful way to deliver H₂O₂ to tissues and thereby selectively kill cells with reduced levels of endogenous antioxidant defences, as may be the case of cancer cells (Chen et al., 2005). This is supported by pharmacokinetics studies with human volunteers showing that plasma AA levels can reach millimolar concentrations immediately after the intravenous administration of such high doses of vitamin C (Padayatty et al., 2004). A rapid decrease in plasma AA is observed thereafter. AA disappearance is thought to occur mainly by efficient renal filtration and excretion, but AA oxidation may also have a significant contribution, as supported by the presence of

AFR in the circulating blood of rats (Wang et al., 1992) and humans (Galley et al., 1996) given a high intravenous dose of AA. In theory, the oxidation of AA in the presence of oxygen can either occur spontaneously or be catalysed by transition metal ions. The former involves the one-electron oxidation of ascorbate anion by molecular oxygen, whereas the latter involves the reaction of oxygen with a metal-ascorbate complex (Khan and Martell, 1967). Chelating agents were shown to slow the oxidation of AA catalysed by iron (DTPA or DFO) or copper (DTPA) in phosphate buffers at neutral pH. Therefore, it was proposed that the auto-oxidation of AA in solution is a consequence of trace levels of transition metal ion (iron and to a lower extent copper) contamination in most solutions employed for research (Buettner, 1988) and would be of limited physiological significance, as transition metals are efficiently sequestered in biological fluids from healthy individuals (Miller et al., 1990). However, our results showed that the genotoxicity of AA could not be blocked by the addition of the metal chelators apo-Tf, DFO, FZN or DTPA. It is worth noting that the endocytosis of DFO and apo-Tf is inhibited at 0 °C and likewise FZN and DTPA are not expected to enter cells during the short incubation on ice due to their hydrophilic nature (Porter, 1989; Parkes et al., 1997). Therefore, these agents would only account for the chelation of extracellular metals in our experimental conditions. Our results suggest that the genotoxicity of AA solutions is due to AA's spontaneous, rather than metal-catalysed, oxidation.

Intriguingly, the addition of apo-Tf and DFO lead to an increase in AA-mediated DNA damage. A possible explanation is that, in the absence of the chelators, some of the H₂O₂ formed from AA auto-oxidation would be lost before entering cells by reacting with iron present in the medium. An excess of apo-Tf or DFO would inhibit the availability of ferric iron and thus spare H₂O₂ loss in the medium. As a consequence, more H₂O₂ would enter cells and damage the DNA in the presence of intracellular reactive iron. A similar effect was reported by Byrnes (1996), who observed that chelation of extracellular iron with non-permeant chelators increased H₂O₂-induced DNA strand breakage in HL-60 cells. This is further supported by the work of Sakagami et al. (1997), who reported that a 1-hour exposure to high (millimolar) concentrations of AA was toxic to HL-60 cells. The addition of micromolar to millimolar concentrations of DFO during the 1-hour exposure did not inhibit but rather enhanced the cytotoxic activity of AA in a dose-dependent way.

Taken together, the two studies mentioned above and our own results show that high concentrations of AA can be toxic even in iron-deficient medium and that iron chelation may enhance the cytotoxicity and genotoxicity of AA.

To summarise, physiological concentrations of AA ($\leq 100 \mu\text{M}$) inhibited the proliferation of HDFs at low-density but were not toxic to confluent cells. On the other hand, at concentrations $\geq 100 \mu\text{M}$, AA caused DNA strand breakage in confluent HDFs in a dose-dependent manner. With our experimental design, we could clearly show that the genotoxic mechanism of AA does not require its intracellular accumulation. Instead, it involves the transient production of H_2O_2 in the cell medium from AA auto-oxidation, the passive diffusion of H_2O_2 through the cell membrane and its reaction with intracellular labile iron, to generate more reactive oxygen species and ultimately damage the DNA. Importantly, the genotoxic effect of AA was independent of the presence of extracellular catalytically active metal ions. Consequently, we anticipate that the pro-oxidant effect of AA described above may occur *in vivo* immediately after intravenous administration of high-dose vitamin C.

Acknowledgements

T. L. D. is grateful to the Fundação para a Ciência e a Tecnologia, Portugal, and the European Social Fund, Third Framework Programme for research support. Work in the laboratory of G. D. D. J. is supported by Cancer Research UK and The Hope Foundation for Cancer Research.

References

- Anderson, D., Yu, T.W., Phillips, B.J., Schmezer, P., 1994. The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay. *Mutat. Res.* 307, 261-271.
- Blanck, T.J., Peterkofsky, B., 1975. The stimulation of collagen secretion by ascorbate as a result of increased proline hydroxylation in chick embryo fibroblasts. *Arch. Biochem. Biophys.* 171, 259-267.
- Blasiak, J., Kowalik, J., 2001. Protective action of vitamin C against DNA damage induced by selenium-cisplatin conjugate. *Acta Biochim. Pol.* 48, 233-240.
- Blasiak, J., Sikora, A., Czechowska, A., Drzewoski, J. 2003. Free radical scavengers can modulate the DNA-damaging action of alloxan. *Acta Biochim. Pol.* 50, 205-210.
- Blasiak, J., Trzeciak, A., Dziki, A., Ulanska, J., Pander, B., 2000. Synergistic effect of vitamin C on DNA damage induced by cadmium. *Gen. Physiol. Biophys.* 19, 373-379.
- Buettner, G.R., 1988. In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *J. Biochem. Biophys. Methods.* 16, 27-40.
- Butler, J.D., Bergsten, P., Welch, R.W., Levine, M., 1991. Ascorbic acid accumulation in human skin fibroblasts. *Am. J. Clin. Nutr.* 54, 1144S-1146S.
- Byrnes R.W., 1996. Evidence for involvement of multiple iron species in DNA single-strand scission by H₂O₂ in HL-60 cells. *Free Radic. Biol. Med.* 20, 399-406.
- Chan, D., Lamande, S.R., Cole, W.G., 1990. Bateman, J. F. Regulation of procollagen synthesis and processing during ascorbate- induced extracellular matrix accumulation *in vitro*. *Biochem. J.* 269, 175-181.
- Chen, Q., Espey, M.G., Krishna, M.C., Mitchell, J.B., Corpe, C.P., Buettner, G.R., Shacter, E., Levine, M., 2005. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. *Proc. Natl. Acad. Sci. USA* 102, 13604-13609.
- Chepda, T.M., Cadau, M., Girin, P., Frey, J., Chamson, A., 2001. Monitoring of ascorbate at a constant rate in cell culture: effect on cell growth. *In Vitro Cell. Dev. Biol. Anim.* 37, 26-30.
- Clement, M.V., Ramalingam, J., Long, L.H., Halliwell, B., 2001. The *in vitro* cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxid. Redox Signal.* 3, 157-163.

- Duarte, T.L., Lunec, J., 2005. Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radic. Res.* 39, 671-686.
- Duthie, S.J., Collins, A.R., 1997. The influence of cell growth, detoxifying enzymes and DNA repair on hydrogen peroxide-mediated DNA damage (measured using the comet assay) in human cells. *Free Radic. Biol. Med.* 22, 717-724.
- Freiberger, H.D., Grove, D., Sivarajah, A., Pinnell, S.R., 1980. Procollagen I synthesis in human skin fibroblasts: effect on culture conditions on biosynthesis. *J. Invest. Dermatol.* 75, 425-430.
- Furumoto, K., Inoue, E., Nagao, N., Hiyama, E., Miwa, N., 1998. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sci.* 63, 935-948.
- Galley, H.F., Davies, M.J., Webster, N.R., 1996. Ascorbyl radical formation in patients with sepsis: effect of ascorbate loading. *Free Radic. Biol. Med.* 20, 139-143.
- Galloway, S.M., Painter, R.B., 1979. Vitamin C is positive in the DNA synthesis inhibition and sister-chromatid exchange tests. *Mutat. Res.* 60, 321-327.
- Halliwell, B., 1989. Protection against tissue-damage in vivo by desferrioxamine - What is its mechanism of action. *Free Radic. Biol. Med.* 7, 645-651.
- Halliwell, B., Gutteridge, J.M.C., 1999. *Free radicals in biology and medicine.* Oxford Science Publications. Oxford.
- Hata, R., Senoo, H., 1989. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissue-like substance by skin fibroblasts. *J. Cell. Physiol.* 138, 8-16.
- Hata, R., Sunada, H., Arai, K., Sato, T., Ninomiya, Y., Nagai, Y., Senoo, H., 1988. Regulation of collagen metabolism and cell growth by epidermal growth factor and ascorbate in cultured human skin fibroblasts. *Eur. J. Biochem.* 173, 261-2677.
- Khan, M.M.T., Martell, A.E., 1967. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I. Cupric and ferric ion catalyzed oxidation. *J. Amer. Chem. Soc.* 89, 4176-4185.
- May, J.M., 1999. Is ascorbic acid an antioxidant for the plasma membrane? *FASEB J.* 13, 995-1006.
- Michiels, C., Toussaint, O., Remacle, J., 1990. Comparative study of oxygen toxicity in human fibroblasts and endothelial cells. *J. Cell. Physiol.* 144, 295-302.

- Miller, D.M., Buettner, G.R., Aust, S.D., 1990. Transition metals as catalysts of "autoxidation" reactions. *Free Radic. Biol. Med.* 8: 95-108.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65, 55-63.
- Murad, S., Grove, D., Lindberg, K.A., Reynolds, G., Sivarajah, A., Pinnell, S.R., 1981. Regulation of collagen synthesis by ascorbic acid. *Proc. Natl. Acad. Sci. USA* 78, 2879-2882.
- Padayatty, S.J., Sun, H., Wang, Y., Riordan, H.D., Hewitt, S.M., Katz, A., Wesley, R.A., Levine, M., 2004. Vitamin C pharmacokinetics: implications for oral and intravenous use. *Ann. Intern. Med.* 140, 533-537.
- Parkes, J.G., Olivieri, N.F., Templeton, D.M., 1997. Characterization of Fe^{2+} and Fe^{3+} transport by iron-loaded cardiac myocytes. *Toxicology.* 117, 141-151.
- Peterkofsky, B., Prather, W., 1977. Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. *J. Cell. Physiol.* 90, 61-70.
- Peterszegi, G., Dagonet, F.B., Labat-Robert, J., Robert, L., 2002. Inhibition of cell proliferation and fibronectin biosynthesis by Na ascorbate. *Eur. J. Clin. Invest.* 32, 372-380.
- Porter, J., 1989. Oral iron chelators: prospects for future development. *Eur. J. Haematol.* 43, 271-285.
- Prasad, K.N., Sinha, P.K., Ramanujam, M., Sakamoto, A., 1979. Sodium ascorbate potentiates the growth inhibitory effect of certain agents on neuroblastoma cells in culture. *Proc. Natl. Acad. Sci. USA* 76: 829-832.
- Re, J.L., Demeo, M.P., Laget, M., Guiraud, H., Castegnaro, M., Vanelle, P., Dumenil, G., 1997. Evaluation of the genotoxic activity of metronidazole and dimetridazole in human lymphocytes by the comet assay. *Mutat. Res.* 375, 147-155.
- Robichova, S., Slamenova, D., 2002. Effects of vitamins C and E on cytotoxicity induced by N-nitroso compounds, N-nitrosomorpholine and N-methyl-N'-nitro-N-nitrosoguanidine in Caco-2 and V79 cell lines. *Cancer Lett.* 182, 11-18.
- Sakagami, H., Satoh, K., 1997. Modulating factors of radical intensity and cytotoxic activity of ascorbate (review). *Anticancer Res.* 17, 3513-3520.
- Sakagami, H., Satoh, K., Fukuchi, K., Gomi, K., Takeda, M., 1997. Effect of an iron-chelator on ascorbate-induced cytotoxicity. *Free Radic. Biol. Med.* 23, 260-270.

- Savini, I., D'Angelo, I., Ranalli, M., Melino, G., Avigliano, L., 1999. Ascorbic acid maintenance in HaCaT cells prevents radical formation and apoptosis by UV-B. *Free Radic. Biol. Med.* 26, 1172-1180.
- Singh, N. P., 1997. Sodium ascorbate induces DNA single-strand breaks in human cells *in vitro*. *Mutat. Res.* 375, 195-203.
- Singh, N.P., McKoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175, 184-191.
- Szeto, Y.T., Benzie, I.F.F., 2002. Effects of dietary antioxidants on human DNA *ex vivo*. *Free Radic. Res.* 36, 113-118.
- Wang, X.Y., Liu, J.K., Yoko, I., Kohno, M., Mori, A., 1992. Direct detection of circulating free-radicals in the rat using electron-spin-resonance spectrometry. *Free Radic. Biol. Med.* 12, 121-126.
- Wee, L.M., Long, L.H., Whiteman, M., Halliwell, B., 2003. Factors affecting the ascorbate- and phenolic-dependent generation of hydrogen peroxide in Dulbecco's modified Eagles medium. *Free Radic. Res.* 37, 1123-1130.
- Winkler, B.S., 1987. *In vitro* oxidation of ascorbic acid and its prevention by GSH. *Biochim. Biophys. Acta* 925, 258-264.
- Wozniak, K., Blasiak, J., 2003. *In vitro* genotoxicity of lead acetate: induction of single and double strand breaks and DNA-protein cross-links. *Mutat. Res.* 535, 127-139.
- Wozniak, K., Blasiak, J., 2004. Vanadyl sulphate can differentially damage DNA in human lymphocytes and HeLa cells. *Arch. Toxicol.* 78, 7-15.

Fig. 1. Effects of AA and AA2P on the viability and proliferation of HDFs. A, Confluent HDFs were incubated with 100 μ M AA or AA2P for 24 hours and viability determined with the MTT assay. Results are the mean $\pm$ SD from 18 replicate wells. The experiment was replicated 3 times with identical results. B, HDFs seeded at low-density were incubated in the presence or absence of 20, 100 or 500 μ M AA or AA2P for 5 days. Medium was changed daily and viable cell number compared with that of control cells. Each value represents the mean $\pm$ SD from 3 independent experiments. Differences between the treated groups and the control group were compared by two-tailed one-way analysis of variance with post-hoc Dunnett's test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ *versus* control; NS, not significant *versus* control.

Fig. 2. Measurement of AA concentration in cell culture medium. AA (100 μ M) was added to low-serum medium (0.5 % FBS) and the concentration of AA was determined at 0, 2, 6, 12 or 24 hours of incubation at 37 °C by HPLC. Results are the mean $\pm$ SD from 3 independent experiments.

Fig. 3. H₂O₂- and AA-mediated formation of DNA damage in HDFs. A, Cells were incubated with different concentrations of H₂O₂ in low-serum medium for 30 minutes on ice and then processed for the comet assay (top). AA solutions were prepared in low-serum medium and incubated at 37 °C in the dark for 1 hour. Confluent cells were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay (bottom). Each value represents the mean $\pm$ SD from 3 independent experiments. B, AA, DHA or AA2P solutions were incubated at 37 °C in the dark for 1 hour. Confluent HDFs were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. Each value represents the mean $\pm$ SD from 3-7 independent experiments. C, AA solutions (500 μ M) were incubated at 37 °C in the dark for different periods of time. Confluent HDFs were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. Each value represents the mean $\pm$ SD from 3 independent experiments.

Fig. 4. Effects of CAT and metal ion chelators on AA-induced DNA damage. AA solutions (500 μ M) were incubated at 37 °C in the dark for 1 hour in the presence or absence of 200 U/ml CAT, 50 μ g/ml apo-Tf or 500 μ M DTPA, FZN, DFO or BIP. Confluent HDFs were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. The last treatment refers to cells that were pre-loaded with 300 μ M DFO for 14 hours and thoroughly washed before exposure to AA. Each value represents the mean $\pm$ SD from 3-10 independent experiments.

Figure 1

Figure 2

Figure 3

Figure 4