As an antioxidant, Vit C is involved in a number of biological processes such as cancer resistance, reduction of DNA damage and chromosomal breakage caused by several carcinogens and other agents (Odin, 1997; Konopacka et al., 2002; Dusinska et al., 2003). However, in experimental studies (Odin, 1997), administration of large amounts of Vit C had a genotoxic effect. More specifically, Vit C has been reported to induce sister chromatid exchanges (SCEs) in CHO cells and human lymphocytes (Weitberg & Weitzman, 1985; Lialiari et al., 1987), chromosomal aberrations, unscheduled DNA synthesis, DNA fragmentation (Rosin et al., 1980) and site-specific DNA cleavage (Kazakov et al., 1988; Wang & Van Ness, 1989). Other studies have also reported that mitomycin C, doxorubicin, cisplatin and hyperoxia, induced chromosomal aberrations and furthermore micronuclei pretreated with certain Vit C concentrations exhibited either protection or induction of chromosomal damage (Gille et al., 1991; Greggi Antunes & Takahashi, 1999; Elsendoorn et al., 2001; Nefic,
These conflicting results motivated us to investigate the potential of Vit C, either alone or in combination with cis-Pt, to induce lesions in cultured human lymphocytes, and certain native nucleic acids.

SCEs have been introduced as a very sensitive method to detect mutagens and/or carcinogens in vitro (Deen et al., 1986; Lialiaris et al., 1992) and in vivo (Mourelatos et al., 1988). It has been shown that the SCE assay has a predictive value, since there is a strong correlation between induction of SCEs and cell death induced by certain drugs (Deen et al., 1986). In the present study, the SCE levels, the Proliferating Rate Index (PRI), and the Mitotic Index (MI) were determined to evaluate the cytogenetic effects of Vit C. In addition, double stranded (dsDNA) and single stranded (ssDNA) DNA, isolated from calf thymus gland, and supercoiled plasmids isolated from E. coli, were used to determine the in vitro effects of Vit C on native nucleic acids (Lialiaris et al., 1992; Pantazaki & Lialiaris, 1999).

MATERIALS AND METHODS

Biochemical studies
Plasmid pTZ18R was isolated from E. coli XL₁, using the alkaline SDS lysis method (Promega Biotech). Native DNA was isolated from calf thymus gland using a standard procedure. Single stranded (ssDNA) was prepared by heating double stranded (dsDNA) at 100°C for 10 min. Two to six μg of each nucleic acid were incubated under the presence of different Vit C concentrations in a final volume of 15 μl. Vit C was present in the form of sodium salt, while all solutions were practically neutral. The biochemical reaction took place at a constant temperature of 37°C, for 30 min and it was terminated by adding 5 μl of loading buffer, consisting of 45% Ficoll (type 400), 1% bromophenol blue and 1% xylene cyanol dissolved in water. The products obtained from Vit C interactions were separated by electrophoresis on a horizontal gel apparatus Biorad (MINI-SUB™ DNA CELL) (1 mg ml⁻¹ ethidium bromide in 40 mM tris-acetate, pH 7.5, 20 mM sodium acetate and 2 mM Na₂EDTA). The samples were allowed to run at a constant current at 5 V cm⁻¹ for approximately 4 hrs. Since ethidium bromide forms a fluorescent complex with DNA, a decreased level of fluorescence signifies diminution of the DNA amount. The gels were visualized under UV light. All assays were performed in duplicate (Lialiaris et al., 1992).

Cytogenetic studies
Heparinized blood samples were obtained from four healthy individuals, none of whom was under any medication treatment or was a smoker. Whole blood cultures were prepared in universal containers by adding 11 drops of whole blood and 5 ml of chromosome medium 1A (Gibco). These were incubated at 37°C for 96 hrs. Cultures were first treated with Vit C (2 mM) at 18th hr after PHA stimulation and/or cis-Pt (10 ng ml⁻¹ final concentration). In order to observe SCEs, 5 mg ml⁻¹ bromodeoxyuridine (BrdU) were then added. Cells were harvested after 2 hrs incubation with colchicine (0.3 mg ml⁻¹ culture medium). Cultures were incubated and manipulated in a dark environment. Slide chromosome preparations were stained using a modified fluorescence plus Giemsa (FPG) technique (Goto et al., 1978). All chemicals were obtained from Sigma, unless otherwise stated.

Scoring was performed in a blind fashion. First, second or third and subsequent cell divisions were identified and counted. Mean SCE values were determined at second division metaphases.

To establish the PRI, 200 cells were counted in each culture of both samples and the following formula was used: \( PRI = \frac{1M_1 + 2M_2 + 3M_3}{100} \), where \( M_1 \) is the percentage of metaphases in the 1st cell division, \( M_2 \) is the percentage of metaphases in the 2nd cell division and \( M_3 \) is the percentage of metaphases in the 3rd and subsequent cell divisions. The MI was determined by counting 3000 activated nuclei in each cell culture of both samples.

Statistical analysis
The evaluation of the MI and PRI was based on the χ²-test. Before the comparison of various treatments using one-way analysis of variance (ANOVA) (followed by Dunkan’s test), logarithmic transformation of the SCEs was performed (Lialiaris et al., 1992).

RESULTS

Direct effect of Vit C on pDNA
One of the purposes of this study was to investigate the in vitro interaction of Vit C with plasmid DNA (pDNA) present in the form of supercoiled and relaxed DNA. The effects of Vit C on the conformational and structural integrity of pDNA are illustrated in Figs 1 and 3. The well-established technique, that we have adopted, enabled us to estimate the conformational and structural changes that took place on
A. Agarose (1%) gel electrophoresis pattern of pTZ18R DNA treated with increasing concentrations of Vit C. Two µg of pDNA were incubated at 37°C for 30 min.
lane 1: λ DNA/HindIII markers: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp.
lane 2: pTZ18R without Vit C treatment (control)
lanes 3-6: pTZ18R treated with 2.5, 5, 10 and 25 mM of Vit C, respectively.

B. Agarose (1%) gel electrophoresis pattern of pTZ18R DNA treated with increasing concentrations of Vit C. Two µg of pDNA were incubated at 37°C for 30 min.
lane 1: λ DNA/HindIII markers: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp.
lane 2: pTZ18R without Vit C treatment (control)
lanes 3-6: pTZ18R treated with 0.5, 1, 2, 3 mM of Vit C, respectively.

C. Agarose (1%) gel electrophoresis pattern of pTZ18R DNA treated with increasing concentrations of Vit C. Four µg of pDNA were incubated at 37°C for 30 min.
lane 2: pTZ18R plasmid without Vit C treatment (control)
lanes 3 and 4: Plasmid pTZ18R treated with 10 mM Vit C
lanes 5: Plasmid pTZ18R linearized after digestion with 5 units of BamHI for 4 hrs at 37°C
lanes 6 and 7: Plasmid pTZ18R treated with 25 mM of Vit C.
pDNA (i.e. from closed circular supercoiled form to an open circular relaxed DNA). More specifically, such changes were quantified by estimating the amount of relaxed DNA. Low Vit C concentrations (1-5 mM) caused a reduction in the amount of relaxed DNA, while a discrete DNA band sizing 1.7 Kb approximately turned up — arbitrarily named a — exhibited a higher electrophoretic mobility than the supercoiled DNA (Fig. 1A, lanes 3 and 4). When high dosages of Vit C were used (10 and 25 mM), a Vit C-induced uncoiling of pDNA was emerged (Fig. 1A, lanes 5 and 6). More specifically, polymeric forms such as catenanes, concatamer forms or chains were observed, exhibiting a delayed electrophoretic mobility when 25 mM of Vit C were used (Fig. 1A, lane 6). The fast-migrating DNA band a was suggested to represent a more compact form of pDNA than the supercoiled DNA or a possible complex of Vit C with the plasmid. Another possible explanation for the emergence of that band could be pDNA cleavage into two specific sites, caused by Vit C.

To confirm the action of low doses of Vit C ranging from 1 to 5 mM on pDNA, a second experiment was conducted and the results are illustrated in Fig. 1B (lanes 4, 5 and 6). The almost total absence of relaxed as well as of linear DNA (along with concurrent emergence of band a, which was mentioned above) encouraged the hypothesis that low Vit C concentrations ranging between 2 and 5 mM elicit a site-specific cleavage within the plasmid, a phenomenon that was not apparent at higher doses (Fig. 1A and C). Even smaller dosages had either no effect at all or they could possibly generate some single stranded nicks, as illustrated in Fig. 1B, lane 3. It is noteworthy, that this distinct band came forward solely at low Vit C dosages (2-5 mM) (Fig. 1A and B).

In addition, in order to confirm the “unwinding” effect of high Vit C concentrations (10 and 25 mM), these experiments were duplicated in particular. At 10 mM of Vit C, it could be observed that pDNA was partially uncoiled, while the intermediate stages of this uncoiling were also apparent (Fig. 1C, lanes 3 and 4). It seems that Vit C does not act by one-hit mechanism under these particular assay conditions, but it causes a stepwise relaxation of the superhelical DNA. According to our experimental proceedings, pDNA was fully relaxed at 25 mM of Vit C dosage (Fig. 1C, lanes 6 and 7).

**Direct effect of Vit C on dsDNA and ssDNA integrity**

Similar *in vitro* studies concerning the effect of Vit C on DNA integrity, revealed a remarkable degradation of both ss and ds calf thymus DNA, when the latter were incubated with Vit C. Furthermore, increasing concentrations of Vit C undoubtedly decreased the initial amount of ss or dsDNA used, suggesting possible numerous scissions or a fragmentation of the DNA (Fig. 2A and B, lanes 2-6 and 2′-6′, respectively). Vit C at 3 mM resulted in an extensive degradation, mainly of the dsDNA, while 6 or 10 mM concentrations lead to an obviously significant ss DNA and dsDNA degradation (Fig. 2, lanes 5, 5′ and 6, 6′, respectively).

**Strategy for detecting site-specific cleavage of supercoiled DNA due to Vit C interaction**

The presence of band a, mentioned above, and our attempt to explain its origin, led us to come up with a strategy to detect whether a site-specific cleavage takes place, indeed. More specifically, in order to investigate that, the following steps were accomplished: i) supercoiled DNA was treated with Vit C under certain conditions that could insure the creation of limited single strand nicks (1 mM of Vit C, in particular), ii) digestion of the treated pDNA took

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**Fig. 2.** Agarose (1%) gel electrophoresis pattern of ssDNA (A) and dsDNA (B) from calf thymus gland treated with different concentrations of Vit C. Two μg of ss or dsDNA, were treated with the indicated concentrations of Vit C at 37°C for 1 hr.

lanes 1 and 1’: ssDNA and dsDNA, respectively without Vit C treatment (control)

lanes 2-6 and 2′-6’: ssDNA and dsDNA, respectively, treated with 1, 2, 3, 6, 10 mM of Vit C.

lane 7: λ DNA/HindIII markers: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp.
place using restriction enzymes such as *BamH1*, so as to obtain linearized DNA, and finally iii) the digested plasmid DNA was incubated with S1 nuclease. S1 nuclease only cleaves the phosphodiester bonds opposite to the existent nick on the complementary strand to generate two DNA fragments. In the case where Vit C initially created single stranded nicks, the size of the double-stranded DNA fragments generated after S1 nuclease treatment, reflects the sites of Vit C scissions.

The results obtained from this experimental procedure, are demonstrated in Fig. 3A, while Fig. 3B outlines the strategy followed. According to this procedure, supercoiled DNA was treated with 1mM Vit C (Fig. 3A, lane 4) in order to create limited nicks on DNA, as previously demonstrated (Fig. 1B, lane 4). The ability of low Vit C concentrations to unwind supercoiled DNA, could be verified by the increase in the amount of relaxed DNA. The distinct band a, also appeared (Fig. 3A, lane 4). Relaxed DNA molecules

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**Fig. 3.**

A. Agarose (1%) gel electrophoresis pattern of pTZ18R DNA treated with increasing concentrations of Vit C. pDNA was linearized by *BamH1* digestion and was sequentially digested with S1 nuclease. Six μg of pDNA were incubated as indicated in each lane.

- lane 1: λ DNA/*HindIII* markers: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp.
- lane 3: pTZ18R plasmid without treatment with Vit C (control)
- lane 4: pTZ18R plasmid treated with 1 mM of Vit C at 37°C for 30 min
- lane 5: pTZ18R plasmid treated as in lane 4 and subsequently digested with 5 units of *BamH1* at 37°C for 30 min
- lane 6: pTZ18R plasmid treated under conditions described as in lane 5 and finally incubated with 50 units of S1 nuclease at 37°C for 30 min.

B. Schematic representation of strategy performed for detecting site-specific cleavage of supercoiled DNA by Vit C.
were linearized using the BamHI restriction enzyme (lane 4), and were then digested by S1 nuclease incubation. The outcome of this procedure is illustrated in Fig. 3A, lane 6.

Since S1 nuclease cleaves ssDNA sequences, it was used to determine whether pretreatment of pDNA with Vit C leads to a site-specific or non-specific DNA cleavage. The former case would generate two segments, visible by electrophoresis as two distinctive bands, while the latter could be visualized as a uniform smear. The results showed that Vit C caused limited but not strictly specific cleavages on the pDNA, as deduced by the large band created with S1 treatment (Fig. 3A). The products of S1 activity were localized in a distinct domain between 1.7 and 0.5 bp and were not extended as a uniform smear in the full length of the gel, neither smaller degradation products were obtained (Fig. 3A, lane 5). A faint band representing the small amount of the unaffected linear DNA also remained.

Cytogenetic studies

The potential ability of Vit C to cause a cytogenetic damage to cultured human lymphocytes, (either alone or in combination with cis-Pt), is demonstrated in Tables 1 and 2. Vit C was studied in low and high concentrations (0.2 mM and 2 mM). Our findings indicate that the low dose did not significantly increase the SCE levels (7.74 SCEs/metaphase), or affected the MI, in comparison to the control (untreated cultures) (6.15 SCEs/metaphase). The combined treatment of 0.2 mM Vit C and cis-Pt (10 ng ml⁻¹) exhibited a statistically significant increase in the SCE frequencies (p < 0.05) compared to the controls, which however was lower than it was expected (EV = 11.24, OV = 10.03, see Table 1). This particular concentration, either alone or in combination, illustrated a degree of cytostaticity (p < 0.05), while the combined treatment exhibited also cytotoxicity (p < 0.05) compared to the control cultures.

The high concentration of Vit C (2 mM) was studied in four experiments using four different donors.

<table>
<thead>
<tr>
<th>Treatment (concentration)</th>
<th>Mean SCEs ± SEM (range of values)</th>
<th>PRI</th>
<th>MI (%e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>6.15 ± 0.37 (1-14)</td>
<td>2.81</td>
<td>40.0</td>
</tr>
<tr>
<td>2. Vit C (0.2 mM)</td>
<td>7.74 ± 0.43 (2-16)</td>
<td>2.72</td>
<td>52.5</td>
</tr>
<tr>
<td>3. Vit C (2 mM)</td>
<td>12.88 ± 0.67 (3-18)</td>
<td>2.50</td>
<td>26.5</td>
</tr>
<tr>
<td>4. cis-Pt (10 ng ml⁻¹)</td>
<td>9.65 ± 0.45 (2-21)</td>
<td>2.73</td>
<td>34.5</td>
</tr>
<tr>
<td>5. Vit C (0.2 mM) + cis-Pt (10 ng ml⁻¹)</td>
<td>10.03 ± 0.46a (5-25) *(EV = 11.24)</td>
<td>2.70b</td>
<td>33.0b</td>
</tr>
<tr>
<td>6. Vit C (2 mM) + cis-Pt (10 ng ml⁻¹)</td>
<td>17.31 ± 0.84a (9-28) *(EV = 16.38)</td>
<td>2.34b</td>
<td>22.0b</td>
</tr>
</tbody>
</table>

The SCEs frequency was based on 30-40 second generation metaphases for each donor and either treatment; for PRI, 200 cells were counted and for MI, 3000 activated lymphocytes, for each donor and either treatment for both indices. The results were based on 2 experiments from 2 donors. PRI and MI comparisons were made by the χ²-test.

*p < 0.05 vs. lines 1, 2 and 3

*b < 0.05 vs. lines 1 and 3
Tables 1 and 2). In all experiments, Vit C was quite genotoxic to the lymphocyte cultures. There was a statistically significant increase compared to the control cultures [(12.88 SCEs/metaphase compared to 6.15 SCEs/metaphase, i.e. a 2-fold increase (Table 1), and 10.08 SCEs/metaphase compared to 7.85 SCEs/metaphase, i.e. a 28.41% increase (Table 2)]. Apart from this, 2 mM of Vit C possessed a higher cytotoxic and cytostatic ability than the \textit{cis}-Pt. Unlike the lower Vit C concentration, the combined administration of Vit C and \textit{cis}-Pt elicited a synergistic effect, inducing high SCE levels (\(p < 0.05\)) and suppressing the PRI (\(p < 0.05\)) and the MI (\(p < 0.05\)) at the same time. In other words, the combined treatment elicited a 2.81-fold increase in SCE levels in the first set of experiments (Table 1) and a 2.22-fold increase in the second set (Table 2).

**DISCUSSION**

There is much controversy about the protective or inducing effect of Vit C on cells treated with genotoxic agents (Rivas-Olnedo \textit{et al.}, 1992; Morales-Ramirez \textit{et al.}, 1998; Donnelly \textit{et al.}, 1999; Konopacka \textit{et al.}, 2002; Dusinska \textit{et al.}, 2003). Tsao \textit{et al.} (1988) in human mammary carcinoma studies, discovered that certain oxidation and degradation products of Vit C are in fact active antineoplastic agents. The ability of antioxidants to protect normal tissues from the damaging effects of cytostatic drugs is attributed to their antimutagenic action, which nevertheless does not inhibit the anti-tumor action of such agents (Elsendoorn \textit{et al.}, 2001). Numerous studies reporting conflicting findings exist up to date. This is mainly due to the variety of concentrations of Vit C used. The present work investigated a wide range of concentrations in an attempt to clarify the correlation of Vit C concentration with its protective or damaging effects. The total absence of relaxed as well as a linear DNA, along with the concurrent emergence of band \(a\), which was mentioned above, encouraged the hypothesis that low Vit C concentrations (from 2 to 5 mM) elicited site-specific cleavage within the plasmid, a phenomenon that was not apparent at higher doses. More specifically, higher concentrations of Vit C caused non-specific nicks leading to plasmid relaxation. In order to examine whether DNA cleavage was site-specific or took place due to structural rearrangements, the effect of Vit C was studied on linear plasmid DNA after enzyme digestion. No site-specific cleavage of linear dsDNA was observed at any Vit C concentration (data not shown). Moreover, when ss or dsDNA substrates were used, it was impossible to observe any specificity, while higher concentrations of Vit C led to partial or complete degradation of the DNA substrate.

<table>
<thead>
<tr>
<th>Treatment (concentration)</th>
<th>Mean SCEs ± SEM (range of values)</th>
<th>PRI</th>
<th>MI (%e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>7.85 ± 0.61 (2-17)</td>
<td>2.41</td>
<td>14.0</td>
</tr>
<tr>
<td>2. Vit C (2 mM)</td>
<td>10.08 ± 0.63 (4-19)</td>
<td>1.98</td>
<td>9.3</td>
</tr>
<tr>
<td>3. \textit{cis}-Pt (10 ng ml(^{-1}))</td>
<td>11.27 ± 0.75 (3-20)</td>
<td>2.29</td>
<td>15.3</td>
</tr>
<tr>
<td>4. Vit C (2 mM) + \textit{cis}-Pt (10 ng ml(^{-1}))</td>
<td>17.39 ± 0.83 (^{a}) (7-31)</td>
<td>1.82(^{b})</td>
<td>7.7 (^{b})</td>
</tr>
</tbody>
</table>

\(^{a}\)EV = 13.50

The SCEs frequency was based on 30-40 second generation metaphases for each donor and either treatment; for PRI, 200 cells were counted and for MI, 3000 activated lymphocytes, for each donor and either treatment for both indices. The results were based on 2 experiments from 2 donors. PRI and MI comparisons were made by the \(\chi^{2}\)-test.

\(^{a}\)\(p < 0.05\) vs. lines 1, 2 and 3

\(^{b}\)\(p < 0.05\) vs. lines 1 and 3
Studies have shown that the ability of ascorbic acid (in mixture with Cu) to cause DNA lesions and protein conformational changes is attributed to the generation of reactive oxygen species (Chiou, 1984). There are reports mentioning the occurrence of a non-enzymatic cleavage of ssDNA at specific sites, involving copper participation in redox reactions (Kazakov et al., 1988). Other studies have suggested the involvement of the DNA secondary structure in site-specific DNA cleavage by ascorbate/Cu(II), while the specificity of DNA lesions in dsDNA has been reported as a negative torsion dependent effect (Wang & Van Ness, 1989). The partially site-specific cleavage generated by Vit C which led to the emergence of band a mentioned above, is probably not related to Cu(II) ions, but it is attributed to other factors responsible for the generation of reactive oxygen species.

Lately, another mechanism was proposed by which Vit C is involved in the interaction of the second-order and higher-order structures of DNA (Yoshikawa et al., 2003). Vit C acting directly on DNA caused lesions and fragmentation leading to the disappearance of nucleic acid bands, almost in the same manner as in the cytogenetic experiments. The effect of Vit C alone or in combination with cis-Pt was studied using the SCEs methodology. Our findings indicated that at low dosages, Vit C exhibited a protective action. On the other hand, the combined, cytogenetic and biochemical findings of this study revealed that Vit C alone, but mainly in combination with cis-Pt, resulted in a serious DNA damage at high concentrations. The different action of Vit C according to its dosage has also been previously reported in human lymphocytes (Lialiaris et al., 1987; Donnelly et al., 1999; Greggi Antunes & Takahashi, 1999; Krishnaja & Sharma, 2003). Whether, Vit C functions in one or the other way, might depend on the concentration of Vit C or cis-Pt and on the length of exposure to these compounds (Rosin et al., 1980).

It has been suggested that the damaging action of Vit C at the molecular and cellular level could be attributed to its interaction with transition metal ions to promote its oxidation, along with hydrogen peroxide production in vitro (Halliwell, 2001). Vit C may also play a role in DNA repair processes in vivo and nucleotide excision repair more specifically (Cooke et al., 1998; Kang et al., 1998).

It is appreciated that, although the concentrations of Vit C used for its interaction with native DNA were relatively high (compared to our in vitro settings), they were still lower than those of the therapeutic dosage used in humans. Certain studies reported the cytotoxic activity of Vit C in a variety of cells and tissues (Rivas-Olnedo et al., 1992) and this is of great importance for the prevention of mutagenicity of Vit C in patients under treatment with this particular vitamin.

We found that DNA treated with high dosages of Vit C is more susceptible to alkylating agents compared with DNA with no Vit C pre-treatment. On the other hand, low concentrations of Vit C have an antimutagenic activity. Similarly, Vit C seems to have a direct effect on native nucleic acids causing site-specific cleavage at low concentrations, while high doses of the anti-oxidant led to partial or complete degradation of the DNA. This study showed the dependence of Vit C damaging effect on concentration. We therefore consider that the clinical use of Vit C in combination with antitumour agents might be of interest in the treatment of human cancer, but cautions must be taken in the concentrations of Vit C used.

ACKNOWLEDGMENTS

The authors are grateful to Prof. DA Kyriakidis for his initial valuable discussion and his propositions for this manuscript.

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