Vitamin C modulates DNA damage induced by hydrogen peroxide in human colorectal adenocarcinoma cell lines (HT29) estimated by comet assay *in vitro*

Renata Kontek[1], Bogdan Kontek[2], Krzysztof Grzegorczyk[3]

[1]Laboratory of Cytogenetics, Department of General Genetics, Molecular Biology and Plant Biotechnology, University of Lodz, Poland
[2]Department of General Biochemistry, University of Lodz, Poland
[3]Department of Endoscopy and One Day Gastroenterology, Wl. Bieganski Memorial Regional Specialist Hospital, Lodz, Poland

Corresponding author:
Dr. Bogdan Kontek
Department
of General Biochemistry
University of Lodz
141/143 Pomorska St
90-236 Lodz, Poland
Phone: +48 42 635 43 36
Fax: +48 42 635 44 84
E-mail:
kontekb@biol.uni.lodz.pl

Abstract

Introduction: Cancer cells, compared to normal cells, are under increased oxidative stress associated with oncogenic transformation, alterations in metabolic activity, and increased generation of reactive oxygen species.
Material and methods: We investigated the ability of vitamin C to reduce the damage induced by hydrogen peroxide, in human colorectal adenocarcinoma cells *in vitro* by the comet assay. Additionally, we measured the kinetics and efficacy of the repair of DNA damage after incubation with vitamin C in the presence of H_2O_2.
Results: The obtained results showed that 1 h pre-incubation with vitamin C and exposure to H_2O_2 for the last 10 min of incubation caused a statistically significant ($p < 0.05$) increase in DNA migration in comet tails in all experimental series. For the 10 µM, 25 µM, 50 µM, 100 µM vitamin C concentrations the levels of DNA damage were as follows: 18.6%, 21.1%, 25.3% and 27.2%, respectively, as compared to the untreated cells (3.26%). However, in comparison with H_2O_2 alone (29.1%), we observed a statistically significant ($p < 0.05$) decrease of the genotoxic effect in HT29 cells induced by H_2O_2 for the two lowest of concentrations of vitamin C: 10 µM and 25 µM. The HT29 cells were able to achieve effective repair of the damaged DNA within 60 and 120 min after incubation with the tested compounds. All the values obtained in the test were statistically significant ($p < 0.05$).
Conclusions: Vitamin C caused a weaker DNA damaging effect of hydrogen peroxide and positively influences the level of oxidative DNA damage in HT29 cells (decrease ~ 30%). We noted that DNA damage was effectively repaired during 120 min postincubation in the tested cells and that oxidative damage was the major type of damage.

Key words: vitamin C, oxidative DNA damage, HT29 cells, comet assay.

Introduction

The effect of ascorbic acid (vitamin C) on cancer treatment has a controversial history [1]. These studies concluded that ascorbic acid treatment brought enhanced quality and prolongation of life. Recently, emerging evidence indicates that ascorbic acid in cancer treatment deserves reevaluation anew [2, 3]. Vitamin C plays an important role in cells; it maintains proper oxidation-reduction potentially participating in neutralization of reactive

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oxygen species (ROS) and reactive nitrogen species (RNS) formed in the course of cellular metabolism or oncogenic transformation [4]. It was shown that ascorbic acid took part in the activation of genes involved in DNA repair, modulating the level of DNA damage in cells exposed to ROS *in vivo* and *in vitro* [5, 6]. The observations suggested that supplementation of vitamin C decreased the endogenous level of oxidative damage (oxidized pyrimidines and strand breaks) in lymphocyte DNA and might help to prevent diseases resulting from tissue damage caused by free radicals [7]. Although numerous studies describe protective action of vitamin C in ROS-exposed cells, reports mentioning genotoxic and pro-oxidative action of ascorbate cannot be disregarded [8]. *In vitro* experiments with cell lines indicate that vitamin C at low concentrations and additionally in the presence of transition metal ions acts as a pro-oxidant and increases the amount of damage to genetic material [9]. In that case the compound enters the Fenton reaction and contributes to the increased amount of damage through the induction of ROS. Mixtures of copper and iron ions with ascorbate cause DNA, lipid and protein damage *in vitro*, stimulating the formation of hydroxyl radical ·OH [10, 11].

In the present study we determined the level of basal DNA damage and the efficacy of DNA repair, recognized in the alkaline comet assay after treatment with vitamin C with or without the presence of 10 μM H_2O_2 during the last 10 min of incubation at 4°C in HT29 cells *in vitro*. The percentage tail DNA (% tail DNA) was evaluated in comparison with untreated cells and cells exposed to H_2O_2. To evaluate oxidatively modified DNA bases, we applied a repair enzyme: formamidopyrimidine-DNA glycosylase (Fpg).

Material and methods

Vitamin C, penicillin-streptomycin, MEM (non-essential amino acid solution), PBS (buffered saline), DAPI (4,6-diamidino-2-phenylindole), bovine serum albumin (BSA), H_2O_2 (hydrogen peroxide), and low melting point (LMP) and normal melting point (NMP) agaroses were obtained from Sigma Chemical Co. Fetal bovine serum (FBS), RPMI 1640 medium, and trypsin-EDTA were supplied by Cytogen (Poland). Fpg enzyme was purchased from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. All other chemicals were of the highest commercial grade available.

The human colorectal adenocarcinoma cell line (HT29) obtained from the American Type Culture Collection (ATCC) was cultured in RPMI 1640 medium supplemented with 10% inactivated FBS, 1% penicillin and streptomycin and 1% MEM non-essential amino acid solution. Cultures of 0.5–1.0 × 10^6 cells/ml were used for all the experiments. Cells were grown at 37°C, 5% CO_2 under standard conditions.

Cell treatment

Vitamin C taken from stock (20 mM) was added to the suspension of HT29 cells to give final concentrations of 10 μM, 25 μM, 50 μM and 100 μM and the cells were incubated with vitamin C for 1 h at 37°C. For the last 10 min of incubation with vitamin C, part of HT29 cells were treated with 10 μM H_2O_2 at 4°C. Each experiment included a positive control, which was only 10 μM H_2O_2 applied for 10 min at 4°C.

Cell viability

Cell viability was determined by trypan blue exclusion assay. After incubation of HT29 cells with described compounds, an equal volume of 0.4% of the trypan blue reagent was added to the cells and the percentage of viable cells was evaluated using a brightfield microscope (NIKON SE). For a single event, 100 cells were analyzed in three independent experiments.

Comet assay

The comet assay has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies [12], genotoxicity testing, and human biomonitoring [13, 14]. Single cell gel electrophoresis (comet assay) is a very sensitive and rapid method for detection of alkali labile sites (ALS), single and double DNA strand breaks and cross-links induced by genotoxic agents [15]. The comet assay was performed under alkaline conditions (pH > 13) according to the procedure of Singh *et al.* [16]. Following 24 h incubation at 37°C, the cancer cells were resuspended in 0.75% low melting point (LMP) agarose in PBS (pH 7.4), and placed on microscope slides precoated with 0.5% normal melting point (NMP) agarose. To prevent additional DNA damage, all the steps were conducted under reduced light or in the dark. Cells were lysed for 1 h at 4°C in a buffer containing 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl, 1% N-lauroylsarcosine sodium, pH 10. Slides were placed in an electrophoresis unit for 40 min to allow DNA to relax in the electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13, at 4°C. Then the slides were subjected to electrophoresis (4°C) for 23 min at an electric field strength of 0.86 V/cm, 25 V, 300 mA. After electrophoresis the slides were neutralized in 0.4 M Tris-HCl (pH 7.5). DNA was stained with DAPI at a concentration of 2 μg/ml. Slides were analyzed using a fluorescence microscope (Olympus BX 60F5, Olympus Optical Co. Ltd.) equipped with a UV-1 filter block at 360 nm connected to the computer-based image analysis system CASP – Comet Assay Software Project by Końca *et al.* [17]. 100 images were randomly selected from each sample, and the percentage of DNA in
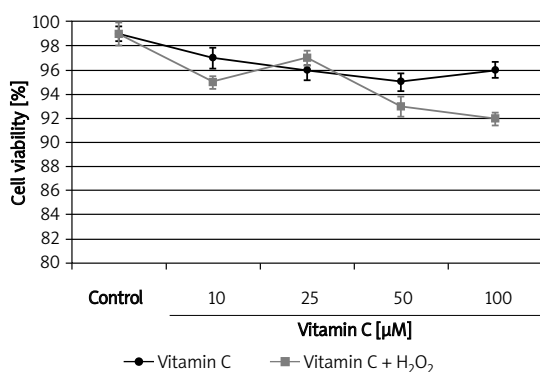
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Figure 1. Effect of 1 h incubation with vitamin C on HT29 cell viability in the absence (black symbols) or in the presence (grey symbols) of 10 μM H$_2$O$_2$ for the last 10 min of incubation at 4°C measured by trypan blue exclusion method
Error bars denote S.E.M.; control – untreated cells

the comet tail (% tail DNA) was measured. The % tail DNA was positively correlated with level of DNA breakage and/or alkali labile sites in the cell and it was negatively correlated with the level of DNA crosslinks [18].

DNA repair

The HT29 cells were incubated with the tested compounds in described systems, and then washed and resuspended in a drug-free growth medium for 60 min and 120 min at 37°C. The comet assay was performed as described above. The repair postincubation (recovery time) was optimized for testing of repair of DNA strand breaks [19].

DNA repair enzyme treatment

After the incubation with tested compounds and lysis, slides were washed three times (5 min, 4°C)
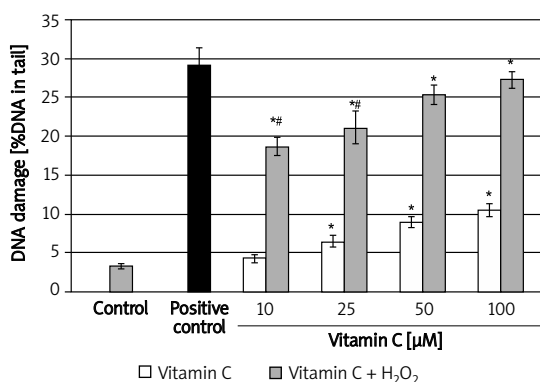

Figure 2. Mean percentage of DNA in the comet tails of HT29 cells exposed to vitamin C for 1 h at 37°C (white bars) as compared with incubation with vitamin C in the presence of 10 μM H$_2$O$_2$ for the last 10 min of exposure (grey bars). The number of cells scored in each individual was 100 and the analysis was repeated three times
*Error bars denote S.E.M., negative control – untreated cells, positive control – 10 μM H$_2$O$_2$ (black bar), *p < 0.05 as compared with control, #p < 0.05 as compared with 10 μM H$_2$O$_2$*

in Fpg buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0) and drained and the agarose was covered with 30 μl of Fpg at 1 μg/ml in the buffer, sealed with a cover glass and incubated for 30 min at 37°C. Further steps were as described above. Fpg is involved in the first step of the base excision repair to remove specific modified bases from DNA and create an apurinic and apyrimidinic site (AP-site), which is subsequently cleaved by its AP lyase activity giving a gap in the DNA strand. The gap can be detected by the comet assay. The enzyme excises mainly 2,6-diamino-4-hydroxy-5-*N*-methyl formamidopyrimidine and 7,8-dihydro-8-oxo-2'deoxyguanine (8-oxo-G) [20]. Hydrogen peroxide was used as an oxidizing agent, which caused DNA damage recognized by Fpg.

Statistical analysis

The values in this study were presented as means ± SEM. from three independent experiments. The differences between the experimental samples and control distribution were evaluated by Student's *t*-test (*p* < 0.05).

Results

Cell viability

The viability of HT29 cells before incubation was 99 ±0.41% (data not shown). The results of trypan blue staining of HT29 cells after 1 h incubation with different concentrations of the tested compounds are shown in Figure 1. We observed that both vitamin C and vitamin C in the presence of 10 μM H$_2$O$_2$ for the last 10 min of incubation caused a slight decrease in the viability of HT29 cells. Vitamin C at the maximal used concentration of 100 μM caused a decrease of HT29 cell viability to 96 ±0.67% whereas vitamin C (100 μM) in the presence of 10 μM H$_2$O$_2$ for the last 10 min of incubation caused a decrease to 92 ±0.52%. After 10 min incubation of HT29 cells with 10 μM H$_2$O$_2$ alone, we observed a decrease in the viability of the cells to 85 ±0.42%.

DNA damage

The mean amounts of DNA in comet tails for HT29 cells exposed to vitamin C for 1 h in the absence and in the presence of 10 μM H$_2$O$_2$ are presented in Figure 2. After incubation of HT29 cells with 10 μM vitamin C no statistically significant increase in DNA in comet tails (4.25 ±0.54%) was observed. At the concentration of 25 μM and 50 μM statistically significant increases in DNA migration in comet tails were observed, by 6.48 ±0.73% and 8.94 ±0.69%, respectively, as compared to the negative control (3.26 ±0.41%). The highest DNA damage, with an increase of 10.52 ±0.9% in comet tails, was noted after the exposure of HT29 cells to vita-

min C at the concentration of 100 μM. The increase in the level of DNA damage during incubation with vitamin C alone was correlated with the increase of the vitamin C concentrations. After the treatment with vitamin C in the presence of 10 μM H_2O_2 during the last 10 min of incubation, a statistically significant (as compared with untreated cells) increase in DNA migration in the comet tails with increasing vitamin C concentrations was noted. For the 10 μM, 25 μM, 50 μM, and 100 μM vitamin C concentrations the levels of DNA damage were as follows: 18.6 ±1.34%, 21.1 ±2.1%, 25.3 ±2.04% and 27.2 ±2.6%, respectively. However, compared with the positive control (10 μM H_2O_2 alone), we observed that 1 h pre-incubation of cells with vitamin C and exposure to H_2O_2 caused a decrease of the genotoxic effect in HT29 cells induced by 10 μM H_2O_2, which evoked strong migration of DNA in comet tails (29.1 ±2.54%) (Figure 2). We noted that in HT29 cells after incubation with the two highest con-

centrations of vitamin C before exposure of H_2O_2, there were no statistically significant differences compared with the positive control. Typical fluorescence microscope images (comet) of the HT29 cells exposed to vitamin C in the presence of H_2O_2 are shown in Figure 3.

DNA repair

We also analyzed kinetics of DNA damage in HT29 cells exposed for 1 h at 37°C to vitamin C in the presence of 10 μM H_2O_2 for the last 10 min of incubation at 4°C after 60 min and 120 min postincubation in medium free of test compounds (Figure 4). The comet tail DNA of the untreated cells was constant, indicating that preparation and subsequent processing of the HT29 cells did not introduce significant damage to their DNA. The HT29 cells exposed to vitamin C with 10 μM H_2O_2 were able to remove damage to their DNA within 60 min, but it was still
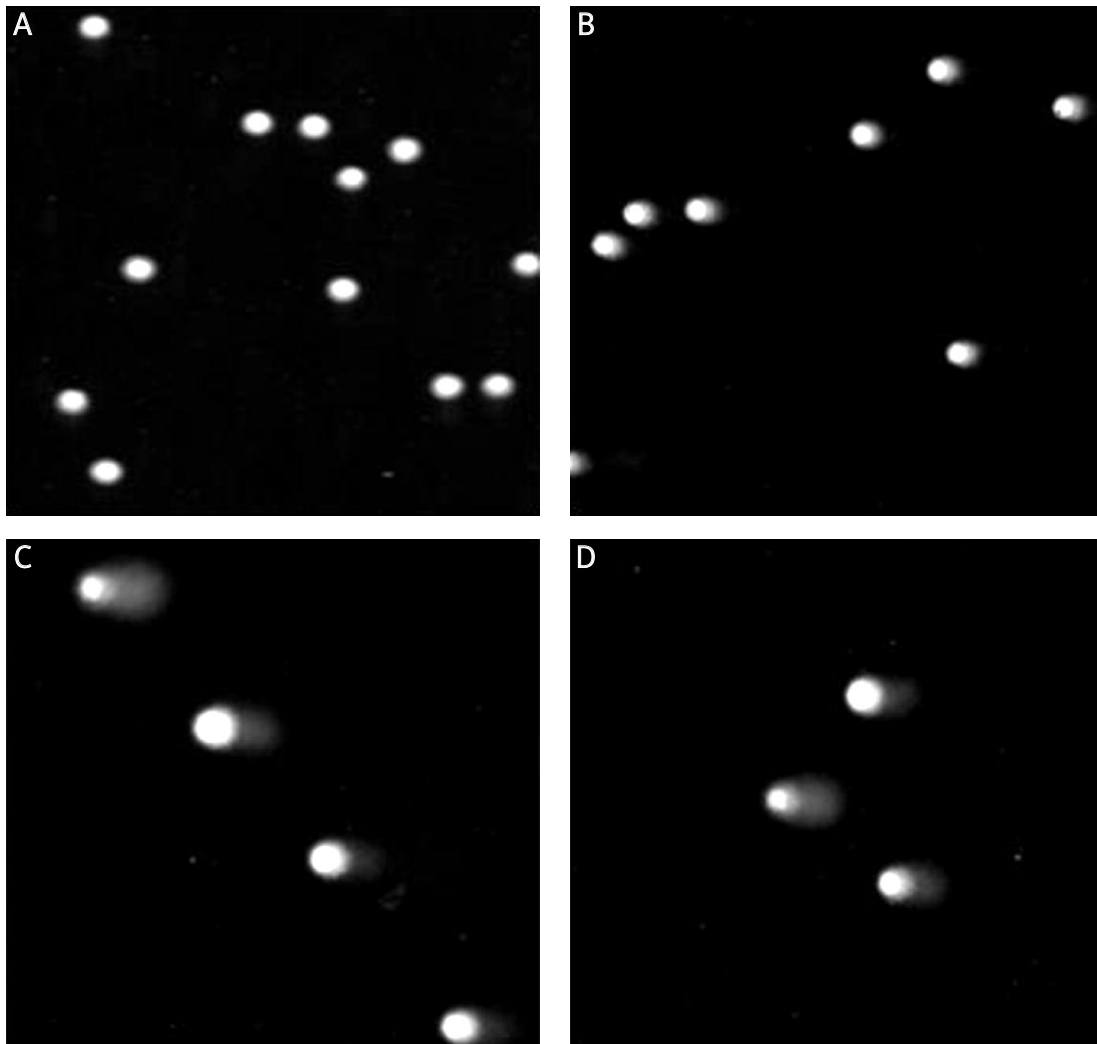

Figure 3. Fluorescence images of comets exposed to vitamin C at the concentration of 25 μM (**B**), 50 μM (**C**) and 100 μM (**D**) for 1 h at 37°C in the presence of 10 μM H_2O_2 for the last 10 min of exposure in HT29 cells (**B–D**) as compared to untreated cells (**A**)
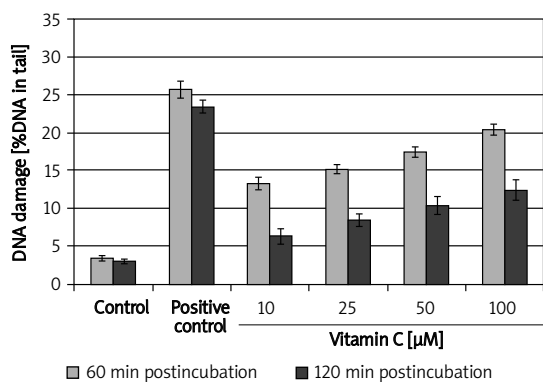
Figure 4. Efficacy of the repair of DNA damage in HT29 cells exposed for 1 h at 37°C to vitamin C in the presence of 10 μM H_2O_2 for the last 10 min of incubation at 4°C after 60 min and 120 min postincubation in medium free of test compounds. The number of cells scored in each individual was 100, and the analysis was repeated three times
Error bars denote S.E.M., control – untreated cells, positive control – 10 μM H_2O_2

Figure 5. Percentage of DNA in the comet tails of HT29 cells exposed for 1 h at 37°C to vitamin C in the presence of 10 μM H_2O_2 for the last 10 min of incubation at 4°C and Fpg enzyme at 1 μg/ml (dark grey bars) as compared with HT29 cells treated with vitamin C in the absence of Fpg (black bars). The number of cells scored in each individual was 100, and the analysis was repeated three times
*Error bars denote S.E.M., control – untreated cells, positive control: 10 μM H_2O_2 (light grey bar) and 10 μM H_2O_2 + Fpg (white bar), *p < 0.05 as compared with 10 μM H_2O_2, **p < 0.05 as compared with 10 μM H_2O_2 + Fpg*

high. The amount of DNA in the comet tails after 60 min of postincubation can be explained by the accumulation of a large number of DNA strand breaks which were not visible directly after the incubation. Effective DNA repair through excision can lead to the formation of secondary DNA strand breaks, visualization of which in the comet test consists in the increase of DNA migration in the tail [17]. During the next hour of incubation (60–120 min) the values decreased to 6.29 ±1.03% for the lowest of the applied concentrations (10 μM), 8.47 ±0.82% for the concentration of 25 μM, 11.34 ±1.2% for the concentration of 50 μM, and 15.38 ±1.32% for the highest of the applied concentrations (100 μM). As compared to the levels of DNA damage following 60 min of postincubation, significant decrease in DNA migration in the comet tails of HT29 cells was noted after 120 min of postincubation. It was demonstrated that DNA damage was effectively repaired during 120 min postincubation in the tested cells.

Figure 5 presents the values of the percentage of DNA in the comet tails in HT29 cells after 1 h exposure to all the tested concentrations of vitamin C and vitamin C in the presence of H_2O_2 for the last 10 min of incubation at 4°C. To allow comparison of the obtained results, a part of HT29 cells was incubated with the tested compounds, but in the absence of Fpg enzyme. The cells exposed to 10–100 μM vitamin C in the presence of 10 μM H_2O_2 and treated with Fpg enzyme showed a higher level of DNA migration in the comet tails as compared to the results obtained for the cells that were not treated with Fpg enzyme. The level of DNA damage (%) increased with increasing concentration of vitamin C in individual trials. The obtained results suggest that oxidative damage was the predominant type of damage among the observed ones.
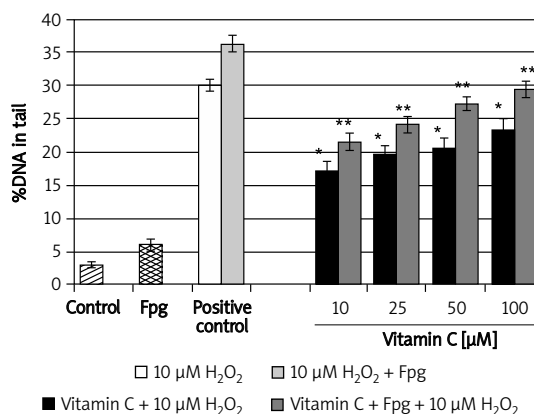
Discussion

Colorectal cancer is a disease having the second highest mortality rate among cancers in developed countries, including Poland. According to the World Health Organization, there are 945,000 newly diagnosed cases each year, 492,000 of which are fatal [21]. The incidence of colorectal cancer is similar for men and women; however, the prevalence of this disease remains higher in men than in women (35% lower in women) [22]. In the present paper we used a human colorectal adenocarcinoma cell line to demonstrate the effect that vitamin C may exert at the level of DNA damage on cancer cells in the presence of hydrogen peroxide. The favorable influence of vitamin C on the human organism has long been known, but its effects on tumor cells and in tumor treatment are controversial. Vitamin C is one of the aqueous-phase antioxidants inhibiting induction of free-radical chain reactions [23]. Interactions of free radicals with DNA can lead to the formation of single-strand or double-strand breaks, formation of AP sites and DNA-protein and DNA-DNA crosslinks, and various chemical modifications of nitric bases. Numerous studies have been concentrated on modulating the influence of antioxidative vitamins, including vitamin C, on the level of DNA damage in cells *in vitro* and *in vivo*, induced by factors triggering free-radical reactions [24].

Experiments in this study indicated that 1 h preincubation with vitamin C in the presence of 10 μM H_2O_2 for the last 10 min of exposure induced DNA damage in HT29 cells and was correlated with the increase of vitamin C concentrations. However,

compared with the positive control, after exposure of HT29 cells to vitamin C in the presence of H_2O_2, we observed a decreased level of DNA damage in HT29 cells induced by H_2O_2. Furthermore, increasing concentrations of vitamin C resulted in increased levels of DNA damage in the comet tails. This fact can be explained by the involvement of high concentrations of vitamin C in the induction of cell death in which DNA breaks were generated. In addition, vitamin C may involved in the Fenton reaction leading to the generation of OH radicals, responsible for DNA oxidative damage in the *in vitro* system [25]. In cancer cells the level of copper ions increases, which can have a toxic influence on the tested cells. It should be noted that the pro-oxidative activity of ascorbate is revealed only in the presence of transition metal ions, and it is a matter of doubt if there are conditions for revealing the pro-oxidative activity *in vivo*, for iron and copper ions are characterized by limited availability to living organism tissues. Ions of those metals are bound by proteins present in cells: ferritin, transferrin and ceruloplasmin [26]. Pro-oxidative activity of low concentrations of vitamin C *in vitro* is associated not only with its involvement in the Fenton reaction, but also in reactions with lipid peroxides. It was shown that vitamin C induces decomposition of lipid hydroperoxides into compounds forming stable adducts with DNA [27]. Also of importance in relation to the purported pro-oxidative activity of vitamin C is that vitamin C has been reported to induce cell death, nuclear fragmentation and internucleosomal DNA cleavage in human myelogenous leukemia cell lines, all in line with the ability of high concentrations of vitamin C to induce apoptosis in various tumor cell lines [28, 29]. Bhat *et al.* [9] observed in their experiments that, at concentrations of 100–200 µM, ascorbic acid is able to generate significant lymphocyte DNA degradation. Although the mechanism of ascorbic acid toxicity is not firmly established, there are results indicating the stimulatory effects on apoptotic pathways, accelerated pro-oxidative damage that cannot be repaired by tumor cells, and increased oxidation of ascorbic acid (at high concentrations in plasma) to a toxic metabolite [30]. Takemura *et al.* [31] observed that a high dose of ascorbic acid induced cell death of four human mesothelioma cell lines. Ascorbic acid mediated cell death was at least due to ROS, especially hydrogen peroxide, accompanied by the disruption of mitochondria structure. Błasiak *et al.* [32] reported that 10 µM vitamin C in the presence of idarubicin (0.001 to 10 µM) (anthracycline anticancer drug) decreased the level of DNA damage in normal lymphocytes *in vitro*. In tumor cells, the presence of vitamin C increased the percentage of DNA in the comet tails – thus it can be considered as a compound increasing the activity of

idarubicin in chemotherapy. In another study, the same author [33], studying the influence of vitamin C and of quercetin on damage induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in human lymphocytes *in vitro*, found that the presence of 50 µM vitamin C inhibits the damaging effect of MNNG, but at high concentrations (250 µM) vitamin C had a pro-oxidative effect, increasing the level of damage in the cells exposed to MNNG. Similar results were reported by Woźniak *et al.* [34], studying cisplatin activity in the presence of vitamin C (50 µM) in lymphocytes and human chronic myelogenous leukemia cells (K562) *in vitro*. In both cell types the vitamin caused an increase in DNA damage over the level observed in the negative control. The target data suggest dual nature of vitamin C activity: antioxidative and pro-oxidative, both for normal cells and cells of neoplastic lines *in vitro*. This is also confirmed by the results obtained in this study. Reports on malignant cancer morbidity among people taking vitamin preparations containing vitamin C are still ambiguous. It was observed that application of vitamin C in various doses not reduce incidents of all types of oxidative DNA damage in human lymphocytes [26].

The results obtained in this study on HT29 cells *in vitro* indicate that vitamin C positively influences the level of oxidative DNA damage in cancer cells. We observed that vitamin C in the presence of 10 µM H_2O_2 caused a slight decrease in the viability of HT29 cells. The presence of vitamin C in tested samples caused a weaker DNA damaging effect of hydrogen peroxide. The cells exposed to tested compounds were able to carry out effective repair of the damaged DNA within 120 min after the end of incubation. The HT29 cells incubated with Fpg enzyme recognizing oxidative DNA damage showed higher level of DNA damage, as compared to the control cells. As expected, the damage was mainly of oxidative nature. The obtained results allow us to conclude that patients suffering from colorectal cancer must have appropriate doses of vitamin C prescribed. Antioxidants have a protective effect for normal tissues by counteracting the unfavorable activity of toxic substances and metabolites present in the human organism and they constitute a part of the antioxidant barrier.

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