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Original scientific article

VITAMINS E AND C EXERT PROTECTIVE ROLES IN HYDROGEN PEROXIDE-INDUCED DNA DAMAGE IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Milev M., M. Angeleska, M. Georgieva, V. Maksimova, M. Janeva, G. Miloshev, T. Ruskovska (2022). *Vitamins E and C exert protective roles in hydrogen peroxide-induced DNA damage in human peripheral blood mononuclear cells*- Genetika, Vol 54, No.3, 1023-1034.

Hydrogen peroxide (H_2O_2) exerts strong oxidative, cytotoxic, and genotoxic effects, whereas vitamins C and E are potent non-enzymatic antioxidants. This study aimed to demonstrate the ameliorative effects of vitamins C and E, individually or in combination, on H_2O_2-induced DNA damage using the alkaline Comet Assay with silver nitrate staining and visual scoring. Trypan blue exclusion assay was used to determine the cytotoxicity of the treatments, whereas alkaline Comet Assay with silver nitrate staining was used to quantify DNA damage. DNA damage was assessed by the method of visual comet scoring and expressed in arbitrary units. Human peripheral blood mononuclear cells (PBMCs) were pretreated with 100 μM vitamin C and E for 30 min, individually or in combination, followed by a treatment with 100 μM H_2O_2 for 30 min. Untreated cells were used as a negative control, whereas cells treated with 100 μM H_2O_2 only were used as a positive control. We observed a considerable H_2O_2-induced DNA damage in the positive control, which was reduced in vitamin-pretreated cells. The combination of vitamins C and E led to the greatest amelioration of DNA damage. In our hands, Comet Assay with silver nitrate staining and visual scoring represents a rapid and reliable method to investigate the protective effects of vitamins C and E on H_2O_2-induced DNA damage.

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INTRODUCTION

Living cells depend on the subtle balance between the levels of oxidants and antioxidants to maintain their integrity. An imbalance in favor of oxidants leads to the oxidation of biomolecules, including DNA. Classically, this imbalance has been referred to as "oxidative stress" (SIES, 1985), although the concept has evolved over the decades (NIKI, 2016; SIES, 2019). Notably, reactive oxygen species (ROS) and related reactive species play a vital role in several cellular physiological functions. For example, ROS are involved in cell signaling, cell division, and protection against infections and carcinogenesis (MITTAL *et al.*, 2014; SIES, 2017; PATTERSON *et al.*, 2019). However, high levels of reactive species leading to oxidative distress are associated with lipid peroxidation, protein oxidation and DNA damage (RUSKOVSKA and BERNLOHR, 2013; NIKITAKI *et al.*, 2015; RUSKOVSKA *et al.*, 2015). On the other side, cellular antioxidant defense comprises enzymes that include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, thioredoxins and others, and non-enzymatic antioxidants such as glutathione, carotenoids and the antioxidant vitamins – vitamin E and C (TRABER and STEVENS, 2011).

Vitamin E is the major lipid-soluble component of the cellular antioxidant system. Its family includes eight chemically distinct forms consisting of an alpha (α), beta (β), gamma (γ) and delta (δ)-tocopherol, and α, β, γ and δ-tocotrienol, all of them referred to as vitamin E (JOSHI and PRATICÒ, 2012). All tocopherols and tocotrienols are potent antioxidants with lipoperoxyl radical scavenging activity. Among all isoforms, α-tocopherol is the most abundant in mammalian tissues, while γ-tocopherol is the primary form of vitamin E in the diet (JIANG *et al.*, 2001). All these molecules are highly lipophilic, and as such, they can interact with bio-membranes and protect them from lipid peroxidation (NIKI, 2014). This role is performed through the structural characteristics of these molecules and the ability to donate phenolic hydrogen to peroxyl radicals, thus forming relatively stable chromanoxyl radicals.

Vitamin C (ascorbic acid) is a potent water-soluble non-enzymatic antioxidant that protects cells against free radicals-induced damage. At physiological pH conditions, vitamin C is mainly an ascorbate anion and donates hydrogen and electrons to an oxidizing system (MEŠČIĆ MACAN *et al.*, 2019). In addition to showing direct antioxidant activity, vitamin C mediates epigenetic regulation in neurodegeneration and cancer (CAMARENA and WANG, 2016) and decreases DNA oxidation through the upregulation of repair enzymes (COOKE *et al.*, 1998).

H_2O_2 is a well-known oxidant that can cross cellular membranes with varying degrees of permeability. This variability may be explained by differences in lipid membrane components, diffusion-facilitating channel proteins, or a combination of both. Homolytic fission of the O-O bond in H_2O_2 produces two hydroxyl radicals. This can be achieved by heat, ionizing radiation, or iron (II) salt, as first observed by Fenton in 1894. Hydroxyl radicals react with extremely high rate constants with almost all biomolecules, including DNA (HALLIWELL and GUTTERIDGE, 1984).

There is evidence that antioxidant vitamins protect cellular integrity from oxidative damage. However, their fundamental functions, particularly the protective effects on the molecule of DNA, remain to be studied in detail. Therefore, we aimed to demonstrate the *in vitro*

effects of antioxidant vitamins E and C, individually and in combination, on basal and H_2O_2-induced DNA damage in human peripheral blood mononuclear cells (PBMCs), using alkaline Comet Assay (single-cell gel electrophoresis) with silver nitrate staining and visual scoring.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were molecular biology or analytical grade. Histopaque-1077, RPMI 1640 cell culture medium, vitamin C, normal melting agarose, Tizma base, sodium chloride, ethylenediaminetetraacetic acid, trichloroacetic acid, glycerol, and Triton X were purchased from Sigma Chemical Co., (St. Louis, MO). Low melting agarose, tungstosilicic acid, zinc sulphate and silver nitrate were purchased from Thermo Fisher Scientific (Waltham, MA). 30% hydrogen peroxide, acetic acid and sodium carbonate were purchased from Merck chemicals (Darmstadt, Germany). We used 97% Trolox as a vitamin E analogue supplied by ACROS organics (Geel, Belgium).

Collection of blood samples

Whole blood (3 mL) was collected in heparinized tubes (BD Vacutainer) from a non-smoking, clinically healthy individual who was not exposed to radiation or taking any drugs, antioxidants, or supplements, after signing informed written consent. Blood collection was conducted in the morning after overnight fasting. The Ethics Committee of the Faculty of Medical Sciences, Goce Delcev University, Stip approved the study protocol (Document No. 2005-162/4).

Isolation of PBMCs

Isolation of cells was performed from fresh blood within an hour of collection. PBMCs were separated from the whole blood according to the method of Boyum (BØYUM 1976). In brief, blood samples were carefully layered over 3 mL of Histopaque-1077 and centrifuged at 400g for exactly 30 min at room temperature without using high acceleration or brakes. The opaque interface was carefully collected and washed with 10 mL of RPMI 1640 cell culture medium. Then it was centrifuged at 250g for 10 min to pellet the cells. This step was repeated twice. The final cell pellet was dissolved in 0.5 mL of cell culture medium.

Cell treatment

H_2O_2 was added to the cell suspension to give a final concentration of 100 μM, and the treatment was conducted for 30 min at 37°C. Untreated cells embedded in the cell culture medium were used as a negative control. To assess the ameliorative effect of antioxidant vitamins E and C, cells were pretreated with a concentration of 100 μM, individually or in combination, for 30 min at 37°C. Before H_2O_2 treatment, the cells were washed twice in a cell culture medium.

The trypan blue dye exclusion method was used to determine cytotoxicity in these experimental conditions (STROBER, 2015). Viable cells were counted with EVE^{TM} Automatic cell counter (NanoEn Tek Inc. South Korea).

Alkaline Comet Assay

After the exposure to the oxidant/antioxidants, the cells were lysed, followed by single-cell gel electrophoresis in alkaline conditions, where damaged DNA forms "comets" consisting of a head and a tail. In brief, after the treatment, the cells were embedded in 0.7% LMA (low melting agarose) and layered on the surface of microscope slides (around 10^6 cells per slide) precoated with 1% NMA (normal melting agarose). The micro-gels were allowed to solidify at 4°C for 15 min in the dark, covered with coverslips. Coverslips were then carefully removed, and slides were immersed in cold lysis solution at 4°C for a minimum of 1 h. Lysis solution was prepared with NaCl (2.5 M), EDTA (100 mM) and Tris base (10 mM), pH = 10. Before use, cold 1% Triton X was added to it. Before electrophoresis, the slides were incubated at 4°C for 40 min in the alkaline denaturing solution (300 mM NaOH, 1 mM EDTA, pH >13), thus allowing DNA to unwind. Electrophoresis was conducted in the same solution at 300 mA current, applying a voltage of 0.8 V/cm across the field for 30 min. After electrophoresis, slides were washed gently 2-3 times for 5 min with neutralizing buffer (0.4 mol/l Tris-HCL, pH = 7.4) and then with deionized water. Then slides were allowed to dry for 1 hour at room temperature.

Silver nitrate staining

The slides were then fixed for 10 min in a fixative solution (1.5% trichloroacetic acid, 5% zinc sulfate and 5% glycerol). After fixation, the slides were washed 3 times with deionized water and left overnight to dry at room temperature. Before silver staining, slides were re-hydrated for 5 min in deionized water. For staining, a fresh solution A (5% sodium carbonate) and solution B (5% sodium carbonate, 0.2% ammonium nitrate, 0.2% silver nitrate, 0.5% tungstosilicic acid, 0.15% formaldehyde) were mixed 3:1 and poured over the slides that were placed in a coloring box. The box was covered to avoid light exposure and placed on a platform swing set in slow motion for 10-20 min to obtain uniform staining. This step was repeated 2-3 times until a greyish color developed on the slides. Then the slides were washed with distilled water 3 times, and the staining was stopped with a treatment of slides for 5 min with 1% acetic acid solution. Finally, the slides were allowed to dry at room temperature (MILEV *et al.*, 2020).

Slide scoring

The silver-stained slides were observed under a light microscope (Leica) with 400× magnification and photographed with a CCD camera. DNA damage was assessed using the visual scoring method published by COLLINS *et al.* (1997). In this approach, the comets were scored in 5 categories from 0 to 4 according to the comet's appearance. One hundred randomly selected nucleoids were analyzed per sample, and the total score was between 0 and 400 "arbitrary units". The objects were chosen without bias and represented the entire gel. The nucleoids from the edges, in the air bubbles, and those that overlapped, were not estimated. Two independent scorers conducted the visual assessment of comets on each slide twice.

Statistical analysis

All results were expressed as mean ± SD. The statistical analysis was conducted using the Excel software with Student's t-test for independent samples, equal variance. Differences were considered significant if $p<0.05$.

RESULTS

Assessment of cytotoxicity

A trypan blue exclusion assay was used to evaluate the cytotoxicity under our experimental conditions. The percentage of trypan blue negative control cells (untreated cells; negative control) in the cell culture medium within 30 min after isolation was 96.5%. Thirty minutes of treatment with vitamins C and E resulted in a small decrease in the percentage of trypan blue negative cells, slightly below 90%. After 30 min of 100 μM H_2O_2 treatment (positive control), the percentage of trypan blue negative cells decreased to 36.7%. Pretreatment of the cells with vitamin C, E or their combination exhibited comparable protective effects, resulting in a percentage of trypan blue negative cells around 57% in all treatments (Fig. 1).

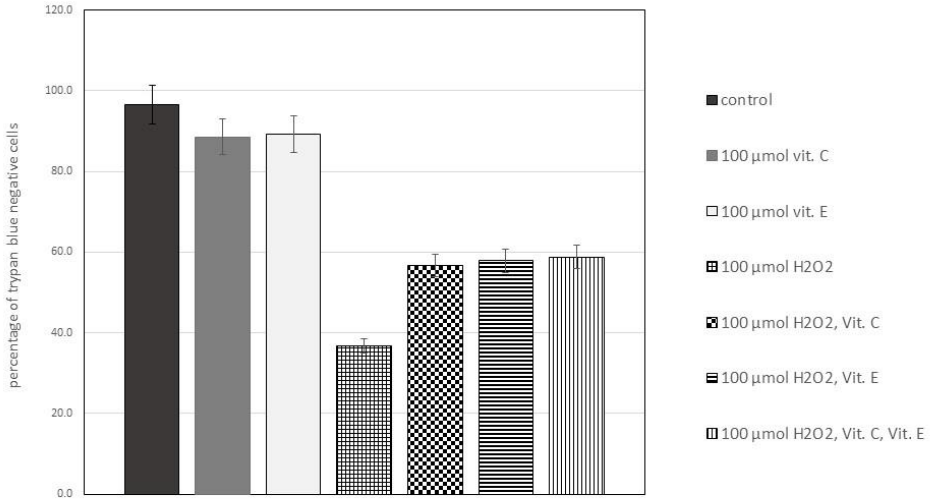

Figure 1. Cytotoxicity was determined with the trypan blue dye exclusion method. Data are presented as mean ± SD. The experiments are conducted in triplicate (n=3).

Treatment of PBMCs with vitamin C or E only, without H_2O_2-induced DNA damage

Alkaline Comet Assay was performed on PBMCs – control and treated with antioxidants. DNA damage observed in the control cells shows the basal (not induced) DNA damage in PBMCs (Fig. 2). Treatment of the cells with vitamin C for 30 min caused a slight and nonsignificant decrease in the level of DNA damage ($p>0.1$). However, the results obtained from the cells treated with vitamin E showed a more intense decrease in basal DNA damage, both compared with the control ($p<0.01$), as well as compared to the vitamin C-treated cells ($p<0.05$). These results are shown in Figure 2.

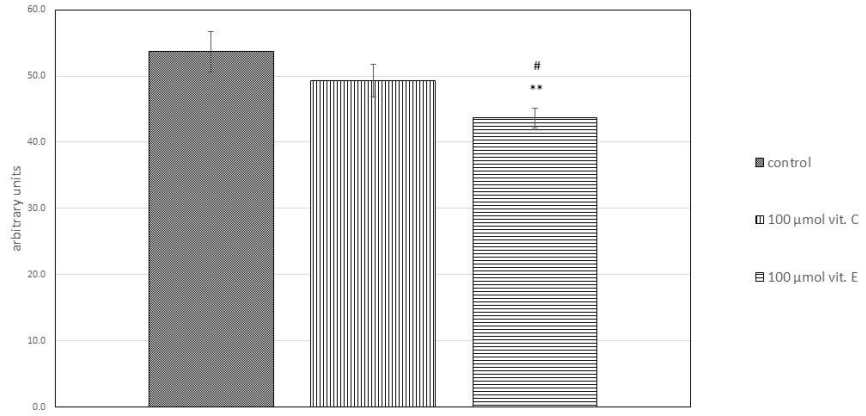

Figure 2. Effect of vitamins E and C on the basal levels of DNA damage in PBMCs. Data are presented as mean ± SD. The experiments are conducted in triplicate (n=3). ** Significance compared to the control, p<0.01. # Significance compared to the 100 µmol vitamin C treatment, p<0.05.

Pretreatment with vitamin C or/and E in PBMCs with H_2O_2-induced damage

PBMCs were treated with 100 µM H_2O_2 for 30 min, leading to considerable DNA damage, as shown in Figure 3. Cells pretreated with vitamin E displayed significant protection from H_2O_2-induced damage (p<0.001). Pretreatment with vitamin C gave a similar but smaller effect (p<0.001). The combination of both vitamins caused the most potent protective effect on H_2O_2-induced DNA damage (p<0.001).

The comparison of different groups of cells showed a statistically significant difference between the pretreatment with vitamin C and the pretreatment with vitamin E (p<0.05), as well as between the vitamin C and the combination of both vitamins (p<0.001) (Figure 3). Representative images from the Comet Assay are presented in Figure 4.

Even by simply observing the pictures presented in Figure 4, one can see that when the cells were pretreated with vitamins, the tail of the comets appeared shorter than in the cells treated only with H_2O_2. This is direct evidence of less DNA damage in pretreated cells. Furthermore, the combined pretreatment of cells with two vitamins led to even shorter tails which resembled the untreated nuclei (compare A with E in Fig.4). This observation proved that the combined pretreatment with both vitamins almost entirely diminished the action of H_2O_2.

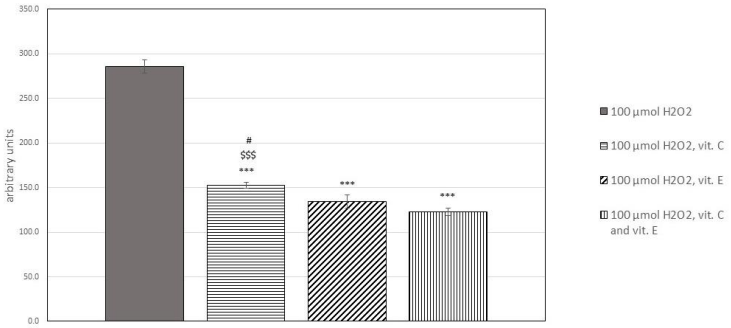

Figure 3. Protective effect of vitamins E and C on H_2O_2-induced DNA damage in PBMCs. Data are presented as mean ± SD. The experiments are conducted in triplicate (n=3). *** Significance compared to the 100 µmol H_2O_2 treatment, p<0.001. $^{\$\$\$}$ Significance compared to the 100 µmol H_2O_2 treatment on vitamins C and E pretreated cells, p<0.001. # Significance compared to the 100 µmol H_2O_2 treatment on vitamin E pretreated cells, p<0.05.

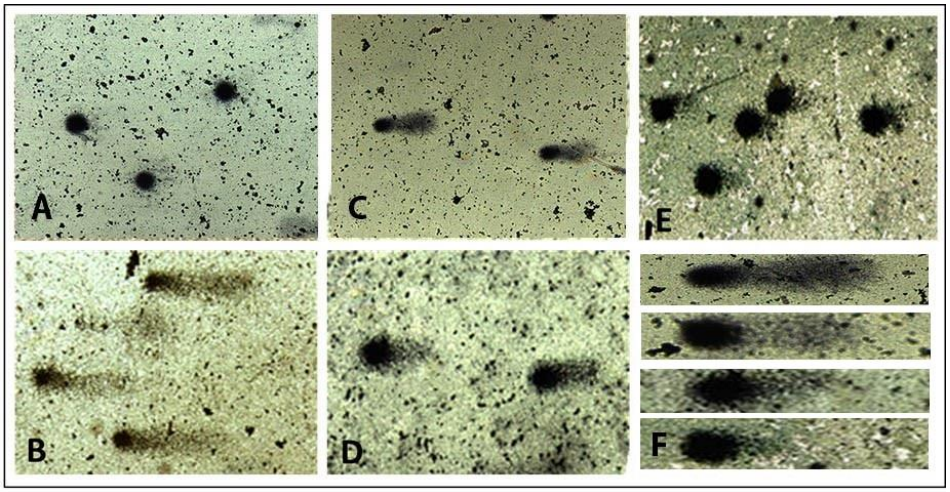

Figure 4. Representative comet images from different types of treatment: A - Untreated control cells; B - Cells treated with 100 µM H_2O_2; C - Pretreatment with100 µM vitamin C + 100 µM H_2O_2; D - Pretreatment with100 µM vitamin E + 100 µM H_2O_2; E - Pretreatment with 100 µM vitamins E and C + 100 µM H_2O_2; F - Size comparison of comets obtained by different treatments (From top to bottom: Cells treated with 100 µM H_2O_2; Pretreatment with100 µM vitamin C + 100 µM H_2O_2; Pretreatment with100 µM vitamin E + 100 µM H_2O_2; Pretreatment with 100 µM vitamins E and C + 100 µM H_2O_2).

DISCUSSION

It is well established that high levels of ROS induce several molecular alterations in the cellular milieu, such as lipid peroxidation and oxidation of proteins and DNA, leading to changes in cell function, morphology, and viability. Hydrogen peroxide is often used as an experimental source of oxygen-derived free radicals (DRIESSENS *et al.*, 2009) and as such, represents a suitable DNA oxidant.

In our experiments, we assessed H_2O_2-induced DNA damage using the method of single-cell gel electrophoresis, also referred to as Comet Assay, as one of the most common methods to quantify DNA damage in eukaryotic cells. This method is also suitable for assessing the antioxidant status of cells by analyzing their resistance to DNA damage induced by ROS. In our experiments, we treated PBMCs with supraphysiological concentrations of H_2O_2, which caused considerable DNA damage. During electrophoresis, damaged DNA migrates in the comet tail. The amount of migrated DNA and its distance from the remaining nucleoid measure DNA damage (COLLINS *et al.*, 2008). The most popular visualization of comets is by staining with DNA-binding fluorescent dyes like DAPI, ethidium bromide or SYBR Green. However, this kind of observation requires a fluorescence microscope, a relatively expensive tool, and a dark room. Moreover, the observation with a fluorescence microscope is limited to approximately two hours. On the contrary, when comets are silver stained, they can be observed for indefinite periods using a simple light microscope. Another advantage of the silver staining method is that the slides can be stored indefinitely at room temperature. Finally, this method is rapid and sensitive and can potentially be used for inter-laboratory comparisons (GARCÍA *et al.*, 2004).

One significant observation of our study is the ability of vitamins C and E to reduce H_2O_2-induced DNA damage, as shown by the lower level of DNA strand breaks detected in the cells pretreated with these vitamins. As a single well-known agent caused the DNA damage in this study, that is, H_2O_2, the comets' shape is very similar; however, there were well-pronounced differences in the length and intensity of the comets' tails (Fig.4) (GEORGIEVA *et al.*, 2015). These differences are most likely due to the different levels of protection that vitamins C and E can exert when used individually or in combination. Our study also shows a positive effect of *in vitro* treatment with vitamins C and E on the level of basal DNA damage in PBMCs.

In our experimental setting, vitamin E offered better protection to PBMCs than vitamin C. This finding can be explained by the lipophilic nature of vitamin E, which enables its antioxidant activity in the membranes (WANG and QUINN, 1999). Indeed, the protective role of vitamin E against H_2O_2-induced DNA damage in human PBMCs is closely related to its solid antioxidative capacity. Namely, vitamin E can scavenge lipid peroxyl radicals, thus terminating the propagation step of the chain reaction of lipid peroxidation. In that way, vitamin E prevents generation of highly reactive aldehydes that can oxidize DNA molecules (RUSKOVSKA *et al.*, 2020). This property makes vitamin E a helpful adjunct in anti-cancer treatments that exert a high level of genotoxicity (AL-EITAN *et al.*, 2020).

Vitamin C is also an effective antioxidant with a high potential to protect cells from oxidative damage (RUSKOVSKA *et al.*, 2015). In our experimental setting, however, we found vitamin C slightly less effective than vitamin E in preventing H_2O_2-induced DNA damage. In contrast, the protective effect of combined treatment (vitamin E plus vitamin C) was more pronounced than the individual ones. These findings can be explained by one of the antioxidant

protection mechanisms of vitamin C, which is its ability to regenerate vitamin E from tocopheryl radical. In that way, vitamin C supports the antioxidative role of vitamin E (CHAN, 1993), which is critical for attenuating lipid peroxidation. Although the biological relevance of this interaction is not completely clear (NIKI, 2014), it has been demonstrated in several *in vitro* studies (PACKER *et al.*, 1979; LAMBELET *et al.*, 1985).

Previous *in vitro* studies have well established the efficacy of treatments with vitamins E or C in preventing cellular oxidative damage (RINNE *et al.*, 2000; SHIVA SHANKAR REDDY *et al.*, 2007; FIORE and CAPASSO, 2008), including DNA oxidation (SHARMA AND SHARMA 2012). However, to our knowledge, the combined effect of vitamin C and vitamin E on H_2O_2-induced DNA damage in PBMCs has not been evaluated so far. Our study confirms that H_2O_2, as a potent oxidant, can induce DNA damage, which is facilitated by antioxidant vitamins C and E.

In conclusion, we leveraged the simplicity and versatility of the alkaline Comet Assay with silver nitrate staining and visual scoring to quantitatively assess the protective effect of vitamins C and E on H_2O_2-induced DNA damage. We demonstrated that simultaneous pretreatment with both vitamins leads to better amelioration of DNA damage.

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VITAMINI E I C IMAJU ZAŠTITNU ULOGU KOD OŠTEĆENJA DNK IZAZVANOG VODONIK PEROKSIDOM U MONONUKLEARNIM PERIFERNIM ĆELIJAMA KRVI

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Izvod

Vodonik peroksid (H_2O_2) ispoljava jake oksidativne, citotoksične i genotoksične efekte, dok su vitamini C i E moćni neenzimski antioksidansi. Ova studija je imala za cilj da demonstrira meliorativne efekte vitamina C i E, pojedinačno ili u kombinaciji, na oštećenje DNK izazvano H_2O_2, korišćenjem alkalnog *Comet Assay* sa bojenjem srebrnim nitratom i vizuelnom ocenom. Test isključenja tripan plavog je korišćen za određivanje citotoksičnosti tretmana, dok je alkalni *Comet Assay* sa bojenjem srebrnim nitratom korišćen za kvantifikaciju oštećenja DNK. Oštećenje DNK je izraženo u proizvoljnim jedinicama. Periferne mononuklearne ćelije krvi (PBMC) su prethodno tretirane sa 100 μM vitamina C i E tokom 30 minuta, pojedinačno ili u kombinaciji, nakon čega je usledio tretman sa 100 μM H_2O_2 tokom 30 minuta. Netretirane ćelije su korišćene kao negativna kontrola, dok su ćelije tretirane samo sa 100 μM H_2O_2 korišćene kao pozitivna kontrola. Primetili smo značajno oštećenje DNK izazvano H_2O_2 u pozitivnoj kontroli, koje je smanjeno u ćelijama prethodno tretiranim vitaminima. Kombinacija vitamina C i E dovela je do najvećeg poboljšanja oštećenja DNK. Utvrdili smo da *Comet Assay* sa bojenjem srebrnim nitratom i vizuelnim ocenjivanjem predstavlja brzu i pouzdanu metodu za istraživanje zaštitnih efekata vitamina C i E na oštećenje DNK izazvano H_2O_2.

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