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Maria Janubova, Jozef Hatok, Katarina Konarikova & Ingrid Zitnanova

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γ- and δ-Tocotrienols interfere with senescence leading to decreased viability of cells

Maria Janubova[1] · Jozef Hatok[2] · Katarina Konarikova[1] · Ingrid Zitnanova[1]

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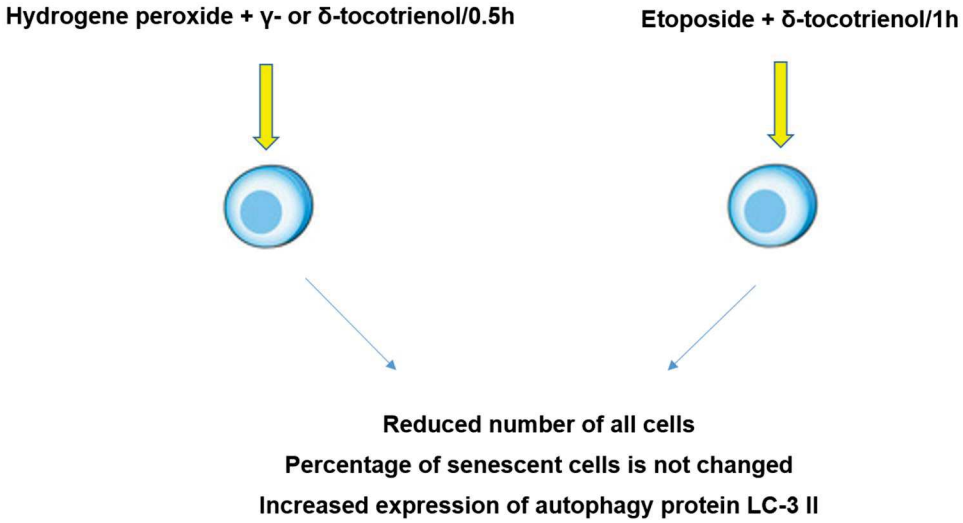
Abstract

Senescence is an irreversible permanent cell cycle arrest accompanied by changes in cell morphology and physiology. Bioactive compounds including tocotrienols (vitamin E) can affect important biological functions. The aim of this study was to investigate how γ- and δ-tocotrienols can affect stress-induced premature senescence. We established two different models of premature stress senescence by induction of senescence with either hydrogen peroxide or etoposide in human lung fibroblasts MRC-5 (ECACC, England). We observed increased percentage of cells with increased SA-β-galactosidase activity, decreased cell viability/proliferation and increased level of p21 in both models. In addition, γ-tocotrienol or δ-tocotrienol (both at concentrations of 150, 200 and 300 μM) were added to the cells along with the inductor of senescence (cotreatment). We have found that this cotreatment led to the decrease of cell viability/proliferation in both models of premature stress senescence, but did not change the percentage of senescent cells. Moreover, we detected no expression of caspase-3 or apoptotic DNA fragmentation in any models of premature stress senescence after the cotreatment with γ- as well as δ-tocotrienols. However, an increased level of autophagic protein LC-3 II was detected in cells with hydrogen peroxide—induced senescence after the cotreatment with γ-tocotrienol as well as δ-tocotrienol. In case of etoposide—induced senescence only δ-tocotrienol cotreatment led to an increased level of LC-3 II protein in cells. According to our work δ-tocotrienol is more effective compound than γ-tocotrienol.

Graphic abstract



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Extended author information available on the last page of the article

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Keywords Senescence · Tocotrienols · Autophagy · Apoptosis · Fibroblasts · MRC-5 cell line

Introduction

Senescence is an irreversible permanent cell cycle arrest accompanied by changes in cell morphology and physiology [1]. Senescence can be induced in cells by various stressors such as DNA damaging agents, oxidative stress, mitochondrial dysfunction and others [1–4]. This type of senescence is called stress-induced premature senescence [1]. The other type of senescence is replicative senescence which is a result of the fact that cells can replicate only a finite number of times [5].

Among typical hallmarks of senescence are enlarged and flattened morphology, a less regular shape, a larger nucleus [1] increased activity of lysosomal senescence associated β-galactosidase (SA-β-galactosidase) [6] and increased expressions of proteins inhibiting the progression of the cell cycle such as p21 and p16 [1, 7].

It has been shown that senescent cells accumulate in tissues with age and also occur in the affected tissues of patients with age-related diseases [8]. On the one hand senescence serves as an anti-cancer mechanism but on the other hand accumulation of senescent cells in organism during aging has detrimental effects. Accumulation of senescent cells contributes to development of chronic inflammation, age-related diseases and cancer. Moreover, senescent cells can induce senescence in other cells in a paracrine manner [9–11].

Tocotrienols belong to the group of lipid soluble compounds called vitamin E. Vitamin E consisting of α-, ß-, γ- and δ-tocopherols and α-, ß-, γ- and δ-tocotrienols, is essential for humans [12].

The present work studied the effects of tocotrienols on stress-induced premature senescence. We investigated the effects of γ-tocotrienol and δ-tocotrienol on stress-induced premature senescence. We chose γ-tocotrienol and δ-tocotrienol because of their superior bioavailability compared to all tocotrienols used along in healthy human subjects [13].

Materials and methods

Chemicals

Minimum Essential Medium (MEM), RPMI-1640 medium, 1% non-essential amino acids, 1% L-glutamine and penicillin-streptomycin mixture, 10% fetal bovine serum, hydrogen peroxide, etoposide (VP-16), γ- and δ-tocotrienols, Thiazolyl Blue Tetrazolium Bromide (MTT), Senescence Cells Histochemical Staining Kit, Protease Inhibitor Cocktail Set III, EDTA-Free, Triton X-100, proteinase K, RNase A, ethidium bromide and cisplatin were purchased from Sigma (Merck), Germany. DC protein assay kit and Clarify Western ECL Substrate kit were obtained from Bio-Rad, USA. Anti-p21 mouse monoclonal primary antibody, anti-Cas-3 rabbit polyclonal primary antibody and anti-α-tubulin primary monoclonal antibody were obtained from Sigma (Merck), Germany. MAP LC3β mouse monoclonal primary antibody, anti-mouse IgG secondary antibody conjugated to HRP and anti-rabbit IgG secondary antibody conjugated to HRP were obtained from Santa-Cruz, Germany.

Cell culture

MRC-5 human lung fibroblasts (ECACC, England) were cultured in MEM containing 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine and penicilin-streptomycin mixture. Cells were incubated at 37 °C in a 5% CO_2 atmosphere in an incubator. For experiments, cells were seeded on culture dishes or plates in amounts described below. Cells at passage number 10–13 were used.

L1210 mouse lymphocytic leukemia cells (ECACC, England) were cultured in RPMI medium containing 10% fetal bovine serum. Cells were incubated at 37 °C in a 5% CO_2 atmosphere in an incubator. For experiments, cells were seeded on culture dishes as described below.

Induction of stress-induced premature senescence in MRC-5 human lung fibroblasts

Cells were cultured for 24 h and senescence was induced by their treatment with either 100 μM hydrogen peroxide for 0.5 h or 80 μM etoposide for 1 h. Markers of senescence were determined on the 4th day after induction of senescence. Etoposide was dissolved in dimethyl sulfoxide as a stock solution of 40 mM and stored at − 20 °C. Hydrogene peroxide was dissolved in 1xPBS as a stock solution of 100 mM just before using. Control cells were treated with either 1xPBS 1000 × diluted in the medium for 0.5 h or 500 × diluted dimethyl sulfoxide (DMSO) in the medium for 1 h.

Treatment of MRC-5 human lung fibroblasts with tocotrienols

γ- and δ-tocotrienols were dissolved in dimethyl sulfoxide as a stock solution of 150 mM and stored at + 4 °C.

Cotreatment: γ-tocotrienol or δ-tocotrienol both at concentrations of 150, 200 and 300 μM were added to the cells

along with 100 μM hydrogen peroxide for 0.5 h or 80 μM etoposide for 1 h. Markers of senescence as well as other analyses were determined on the 4th day after cotreatment.

Cells treated with dimethyl sulfoxide 500 × diluted in the medium but not treated with tocotrienols were used as control cells.

MTT viability/proliferation assay

Cells were seeded on 96-well plates at a density of 3200 cells/well and treated as described above. On the 4th day after senescence induction thiazolyl blue tetrazolium bromide (MTT) was added to the culture medium. After a 4-h incubation at 37 °C in a 5% CO_2 atmosphere the culture medium containing MTT was removed and 200 μl of DMSO was added to each well. The absorbance was measured at 490 nm with a spectrophotometer BioTek EL808, USA.

SA-β-galactosidase assay

Cells were seeded on 24-well plates at a density of 5000 cells/well (control cells) or 38,000 cells/well (cells with induced senescence). Cells were treated as described above.

Cellular senescence was determined by SA-β-galactosidase staining. SA-β-galactosidase staining was performed using the Senescence Cells Histochemical Staining Kit (Sigma/Merck, Germany) according to the manufacturer's guidelines. The staining was evaluated after 16–18 h incubation at 37 °C in a CO_2-free atmosphere. Cells from 15 different fields were counted. A percentage of blue stained cells (SA-β-galactosidase positive cells) were presented as the percentage of senescent cells.

Western blot analysis

Cells were seeded on culture plates at a density of 2,000,000 cells/100 mm dish and treated as described above. Proteins were isolated from the cells on the 4th day after induction of senescence. L1210 cells were seeded on culture plates at 1,500,000 cells/100 mm dish. After 24 h 6 μM cisplatin diluted in 1xPBS was added to the cells. After 24 h proteins were isolated.

Cells were washed with warm PBS, trypsinized and centrifuged at 700 g. Pellets were washed with cold PBS and centrifuged three times at 700 g and then stored at − 20 °C. Cell lysis was performed within 14 days since preparation. Cells were lysed for 40 min at 4 °C in lysis buffer consisting of 1% SDS, 1% Triton X-100, 100 mM NaCl, 1 mM EDTA, pH 6.9, 50 mM Tris pH 8.0 and inhibitors of proteases (1:200), then centrifuged for 20 min at 21 000 g.

Protein concentrations were determined by DC protein assay kit (Bio-Rad, USA) according to the manufacturer´s protocol.

5 or 10 μg of proteins were separated by 12% or 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis for 50 min at 150 V and then transferred to nitrocellulose membranes for 1 h at 80 V. Membranes were blocked with 5% skimmed milk for 1 h and then incubated with primary antibodies. The mouse or rabbit primary antibodies against p21 (1:1000), Cas-3 (1:100), LC-3 (1:1000) and α-tubulin (1:4000) were used in this study. Finally, the membranes were treated with an anti-mouse or anti-rabbit IgG secondary antibody (1:5000). The blots were developed using Clarify Western ECL Substrate kit (Bio-Rad, USA) according to the manufacturer´s protocol. The quantification of relative protein expression was done using software Image Lab 5.0.

Horizontal gel electrophoresis

MRC-5 cells were seeded on culture plates at a density of 150,000/60 mm dish (control cells) or 1,000,000 cells/60 mm dish (cells with induced senescence) and treated as described above.

L1210 cells were seeded on culture plates at the density of 800,000 cells/60 mm dish. After 24 h 6 μM cisplatin diluted in 1xPBS was added to the cells for 24 h.

On the 4th day after the induction of senescence (MRC-5 cells) or 24 h after adding cisplatin (L1210) cells were washed with a warm PBS, trypsinized and centrifuged at 700 g. Pellets were washed with the cold PBS and centrifuged three times at 700 g and then stored at -20 °C. Cell lysis was performed within 14 days since preparation. Cells were lysed in lysis buffer consisting of 0.5% Triton X-100, 10 mM Tris, 10 mM EDTA pH = 8.0 and 0.1% proteinase K for 1 h at 37 °C and 10 min at 70 °C. After that 2.5 μl of RNAse A (0.4%) was added to the samples and then the samples were incubated for 1 h at 37 °C.

The samples were separated by horizontal gel electrophoresis for 4 h at 40–60 V and then analyzed by UV transilluminator Vilber Lourmat, France at 254 nm. 0.1% ethidium bromide (1:2000) was added to the gel.

Statistics

The results are presented as the mean ± SD of a minimum of three independent experiments. Statistical significance was determined by the One-way ANOVA with a Bonferroni correction or by the Student´s *t*-test when appropriate. A value of $p < 0.05$ was considered significant.

Results

Activity of SA-β-galactosidase under stress inducing premature senescence in MRC-5 human lung fibroblasts

Senescence was determined by the detection of SA-β-galactosidase positive cells since increased activity of SA-β-galactosidase is one of the typical hallmarks of senescence [6]. To induce premature senescence we treated MRC-5 human lung fibroblasts with either 100 μM hydrogen peroxide for 0.5 h or 80 μM etoposide for 1 h. Significantly higher number of SA-β-galactosidase positive cells was observed among cells treated with 100 μM hydrogen peroxide (84.05 ± 5.45%) (Fig. 1a) or cells treated with 80 μM etoposide (90.36 ± 2.22%) (Fig. 1a) compared to control cells (2.63 ± 1.23% or 1.77 ± 0.58%, respectively) (Fig. 1a).

Cell viability/proliferation under stress inducing premature senescence in MRC-5 human lung fibroblasts

To confirm senescence we also performed MTT viability/proliferation assay. Cell viability/proliferation was significantly lower in cells treated with 100 μM hydrogen peroxide (32.34 ± 5.95%) compared to the untreated control (Fig. 1b). Similarly, treatment of cells with 80 μM etoposide led to significant decrease of cell viability/proliferation (39.79 ± 5.81%) compared to the untreated control (Fig. 1b). The cell cycle arrest is one of the markers of senescent cells [1]. Consequently, senescent cells are unable to proliferate.

p21 protein level under stress inducing premature senescence in MRC-5 human lung fibroblasts

Further, we performed western blot to detect expression of p21 on protein level since increased expression of p21 is another hallmark of senescence. We detected significantly increased expression of p21 in cells treated with 100 μM hydrogen peroxide or 80 μM etoposide compared to control cells (Fig. 1c). The expression of p21 in cells treated with 80 μM etoposide was 2.95 ± 0.42 fold higher than the expression of p21 in control cells, while the expression of p21 in cells treated with 100 μM hydrogen peroxide was 4.44 ± 0.63 fold higher than the expression of p21 in control cells (Fig. 1d).

Effect of γ- or δ-tocotrienols cotreatment on cell viability/proliferation under stress inducing premature senescence in MRC-5 human lung fibroblasts

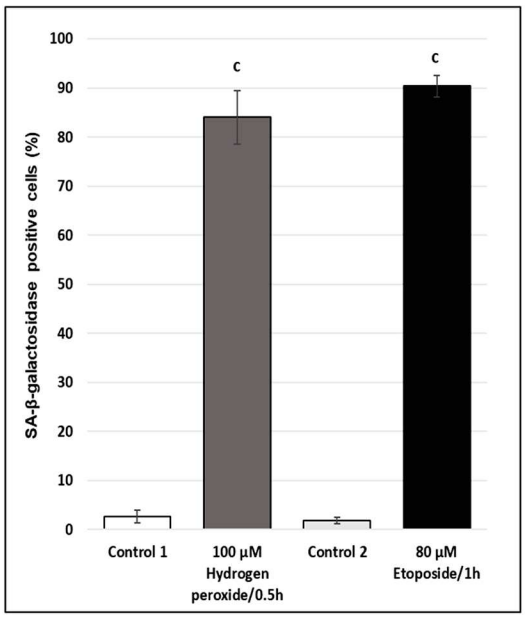
At first we investigated effect of γ-and δ-tocotrienols, both at concentrations of 150, 200 and 300 μM, on cell viability/proliferation in MRC-5 human lung fibroblasts in which senescence was not induced. Cells were treated with tocotrienols for either 0.5 h or 1 h (Fig. 2a, b). We did not observed a significant decrease of cell viability/proliferation neither in cells treated with γ-tocotrienol nor in cells treated with δ-tocotrienol.

However, cotreatment with γ-tocotrienol led to the large decrease of cell viability/proliferation (to 63.86 ± 9.35%, 62.62 ± 9.3% and 47.35 ± 12.46%, respectively) in cells in which senescence was induced by hydrogen peroxide compared to PD control cells (Fig. 2c). On the other hand, cotreatment with γ-tocotrienol did not affect cell viability/proliferation much in cells in which senescence was induced by etoposide compared to ED control cells (Fig. 2d). Cotreatment with δ-tocotrienol also led to the strong decrease of cell viability/proliferation (to 44.85 ± 12.46%, 33.33 ± 9.35% and 22.12 ± 6.23%) in cells in which senescence was induced by hydrogen peroxide compared to PD control cells (Fig. 2c). In cells in which senescence was induced by etoposide only cotreatment with 300 μM δ-tocotrienol strongly decreased cell viability/proliferation (to 51.77 ± 7.58%) compared to ED control cells (Fig. 2d).

Effect of γ- or δ-tocotrienols cotreatment on activity of SA-β-galactosidase under stress inducing premature senescence in MRC-5 human lung fibroblasts

We investigated the effects of 300 μM γ-tocotrienol or 300 μM δ-tocotrienol cotreatment on activity of SA-β-galactosidase under stress inducing premature senescence in MRC-5 human lung fibroblasts. We observed that cotreatment of 100 μM hydrogen peroxide with 300 μM γ-tocotrienol or 300 μM δ-tocotrienol led to the decrease in the number of all cells compared to PD control cells (Fig. 3a). However, the percentage of senescent cells was reduced only mildly compared to the percentage of senescent cells in PD control cells (Table 1).

Cotreatment of 80 μM etoposide with 300 μM δ-tocotrienol led to the large decrease in the number of all cells compared to ED control cells (Fig. 3b), but the percentage of senescent cells did not change significantly compared to the percentage of senescent cells in ED control cells (Table 1).



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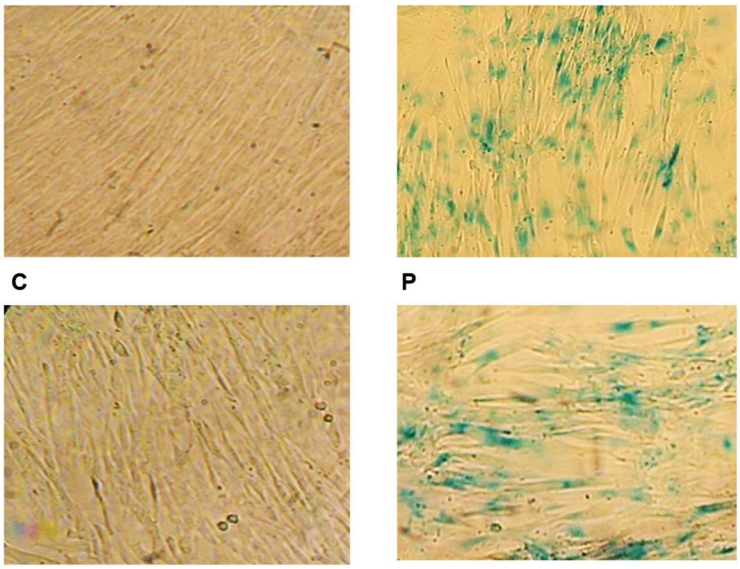

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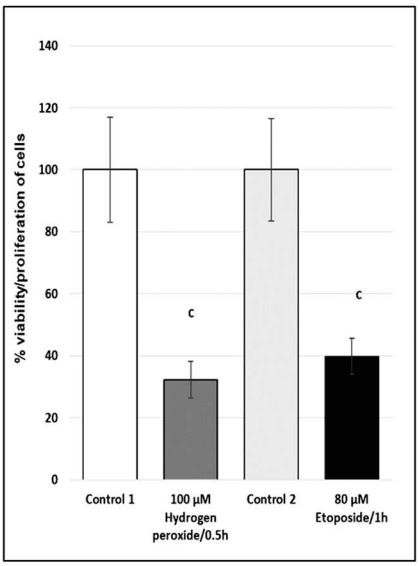

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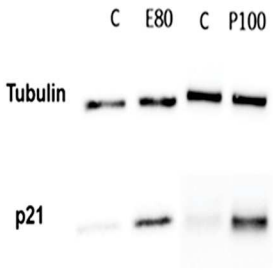

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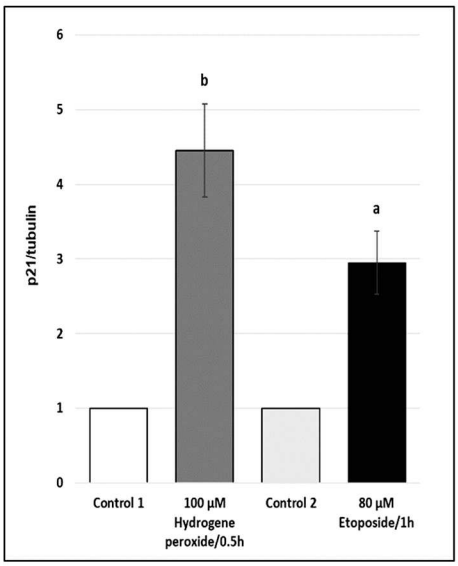

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Fig. 1 **a, b** Activity of SA-β-galactosidase under stress inducing premature senescence in MRC-5 human lung fibroblasts **c** Cell viability/proliferation under stress inducing premature senescence in MRC-5 human lung fibroblasts **d** p21 protein level under stress inducing premature senescence in MRC-5 human lung fibroblasts **e** The relative expression of p21 MRC-5 human lung fibroblasts were treated with either 100 µM hydrogen peroxide for 0.5 h or 80 µM etoposide for 1 h. The protein level of p21 was evaluated by western blot with anti-p21 monoclonal antibody. Tubulin served as a loading control. *C* control cells not treated with hydrogen peroxide or etoposide (10 µg); E80–cells treated with 80 µM etoposide for 1 h (10 µg); P100-cells treated with 100 µM hydrogen peroxide for 0.5 h (10 µg); The values are means ± SD of three independent experiments (*n* MTT test = 16–18; *n* SA-β-gal = 15; *n* WB = 3); Statistical significance was determined by the Student´s *t*-test. a-$p < 0.05$; b-$p < 0.01$; c-$p < 0.001$; **b** 100 × magnification

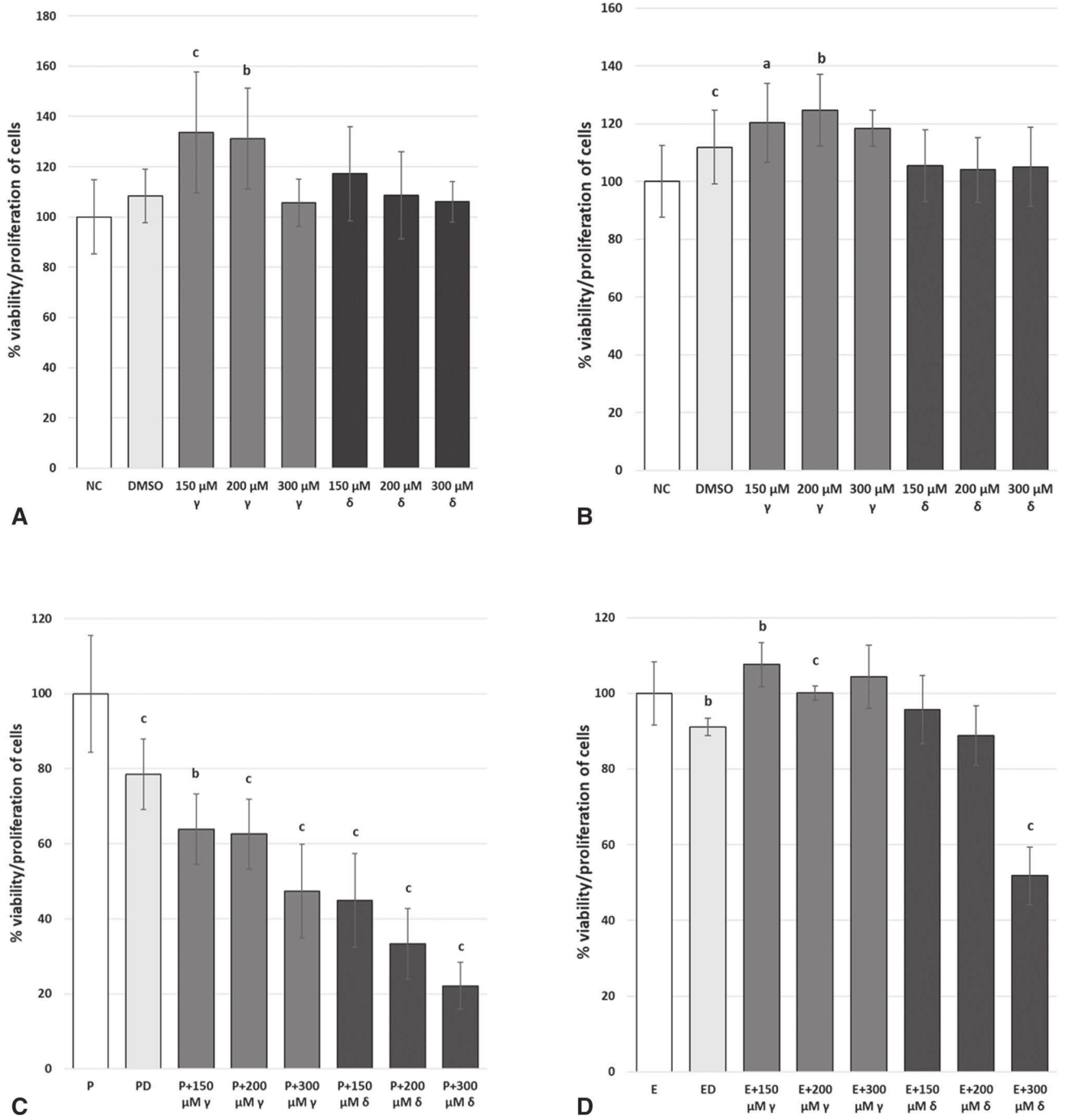


Fig. 2 **a**, **b** Effect of γ- and δ-tocotrienols on cell viability/proliferation in MRC-5 human lung fibroblasts MRC-5 human lung fibroblasts treated with γ-tocotrienol or δ-tocotrienol at concentrations of 150, 200 and 300 µM for 0.5 h **a** or 1 h **b** *NC* normal cells (without tocotrienols and dimethyl sulfoxide); DMSO control–cells treated with dimethyl sulfoxide 500x (0.5 h/1 h) diluted in the medium but not treated with tocotrienols **c**, **d** Effect of γ-and δ-tocotrienol cotreatment on cell viability/proliferation under stress inducing premature senescence in MRC-5 human lung fibroblasts P–cells treated with 100 µM hydrogen peroxide for 0.5 h**;** E—cells treated with 80 µM etoposide for 1 h. PD/ED—untreated control—cells treated with dimethyl sulfoxide 500 × diluted in the medium but not treated with tocotrienols. The values are means ± SD of three independent experiments ($n = 9$–30). Statistical significance was determined by the One-way ANOVA with the Bonferroni correction. NC vs. DMSO; DMSO vs. γ; DMSO vs. δ. P vs PD; PD vs Pγ; PD vs Pδ; E vs ED; ED vs. Eγ; ED vs Eδ. a-$p < 0.05$; b-$p < 0.01$; c-$p < 0.001$

γ- or δ-tocotrienols cotreatment did not lead to the expression of caspase-3 under stress inducing premature senescence in MRC-5 human lung fibroblasts

We hypothesized that γ-tocotrienol or δ-tocotrienol could lead to the decrease of the number of cells including senescent cells by apoptosis. We investigated the effects of 300 μM γ-tocotrienol or 300 μM δ-tocotrienol cotreatment on the expression of caspase-3. We performed the western blot and immunodetection of caspase-3. However, we did not detected expression of caspase-3 neither after cotreatment with γ-tocotrienol nor δ-tocotrienol (Fig. 4a).

γ- or δ-tocotrienol cotreatment led to an increased level of the autophagic protein LC-3 II under stress inducing premature senescence in MRC-5 human lung fibroblasts

We also studied expression of the autophagic protein LC-3 after cotreatment with 300 μM γ-tocotrienol or 300 μM δ-tocotrienol as we supposed that γ- or δ-tocotrienol could lead to changes in autophagy since increased as well as decreased autophagy may be associated with cell death [14]. We found that cotreatment with 300 μM γ-tocotrienol led to the increased level of LC-3 II protein in cells in which senescence was induced by 100 μM hydrogen peroxide (Fig. 4b, PG) and cotreatment with 300 μM δ-tocotrienol led to the increased level of LC-3 II protein in cells in which senescence was induced by 100 μM hydrogen peroxide (Fig. 4b, Pde) as well as in cells in which senescence was induced by 80 μM etoposide (Fig. 4b, Ede).

γ- or δ-tocotrienol cotreatment did not lead to apoptotic DNA fragmentation under stress inducing premature senescence in MRC-5 human lung fibroblasts

Finally, we investigated apoptotic DNA fragmentation by horizontal gel electrophoresis after cotreatment with 300 μM γ-tocotrienol or 300 μM δ-tocotrienol. We did not detect the apoptotic DNA fragmentation either after cotreatment with γ-tocotrienol or δ-tocotrienol in cells in which senescence was induced by hydrogen peroxide (Fig. 5). Similarly, cotreatment with δ-tocotrienol in cells in which senescence was induced by etoposide did not lead to the apoptotic DNA fragmentation (Fig. 5).

Discussion

In this study we investigated effect of γ-tocotrienol and δ-tocotrienol on the stress-induced premature senescence in human lung fibroblasts.

The first aim of our work was the induction of premature stress-induced senescence in cells in in vitro conditions using two different stressors hydrogen peroxide or etoposide on MRC-5 human lung fibroblasts. Fibroblasts including MRC-5 human lung fibroblasts are typical cells used to study all types of senescence [2, 3, 9, 15]. Addition of hydrogen peroxide to cells leads to an increased level of reactive oxygen species and to the oxidative damage to various macromolecules, whereas etoposide causes DNA double strand breaks by the inhibition of DNA topoisomerase II [16–18]. In our two senescence model systems we detected three typical hallmarks of senescence [1, 6, 7]-increased activity of SA-β-galactosidase, decreased proliferation of cells and increased expression of p21.

Hydrogene peroxide is able to induce senescence in various types of cells (human fetal astrocytes; HUVEC-human vein endothelial cells; mesenchymal stem cells; adipocytes). Similarly, etoposide induces senescence in different cell types (IMR90-human diploid fibroblasts; BJ fibroblasts; nenonatal human keratinocytes) [15, 19–23].

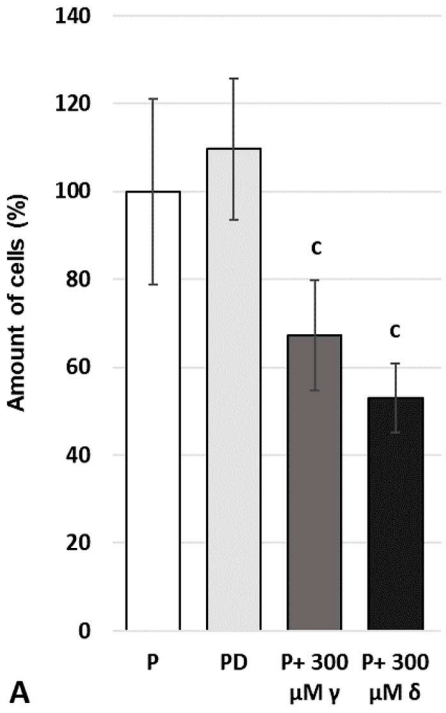
These cells also showed increased SA-β-galactosidase activity and/or increased p21 expression and/or decreased cell viability/proliferation as well as other markers of senescence (e.g. formation of senescent associated heterochromatin foci–SAHF, increased expression of p16, increased production of reactive oxygen species) [15, 19–23].

The second aim of our work was the investigation of γ-and δ-tocotrienol effects on stress-induced premature senescence in our two models of senescence.

Our experiments have revealed that cotreatment with γ- or δ-tocotrienol is accompanied by the decrease of cell viability/proliferation in cells in which senescence was induced by hydrogen peroxide whereas only cotreatment with δ-tocotrienol decreased cell viability/proliferation in etoposide–induced senescence.

Previously, it has been reported that α-tocopherol may also act as a pro-oxidant via the alpha-tocopheroxyl radical [24]. Therefore, tocotrienols might also show the pro-oxidant activity and enhance the pro-oxidant effect of hydrogen peroxide in cells in which senescence was induced by hydrogen peroxide. Like tocopherol radicals, tocotrienol radicals (higher concentrations) could react with polyunsaturated fatty acids leading to the formation of lipoperoxyl radicals and lipoperoxides and propagation of radical reactions.

Our analysis of SA-β-galactosidase activity after cotreatment with γ-tocotrienol as well as δ-tocotrienol has confirmed the decrease in the number of all cells but the



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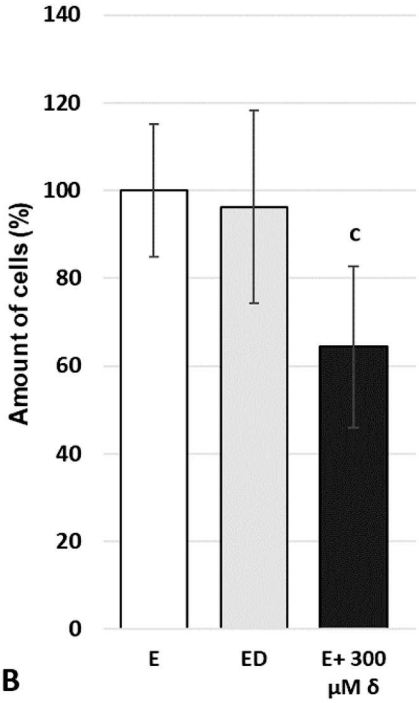

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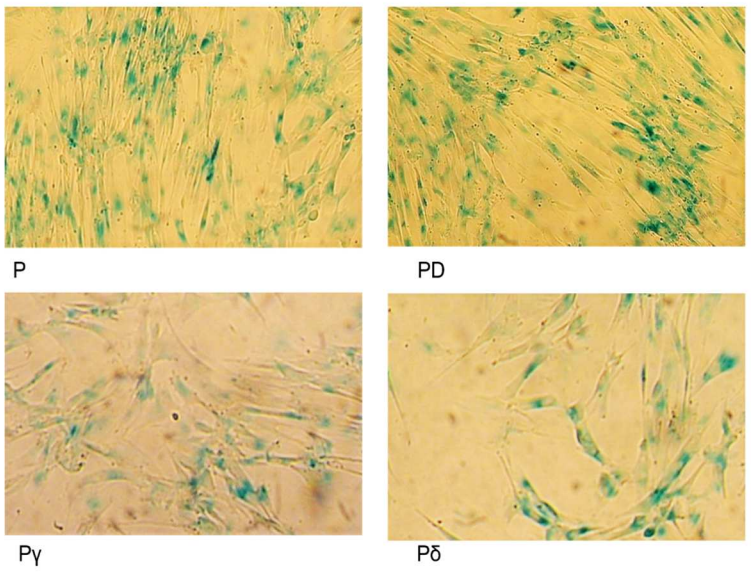

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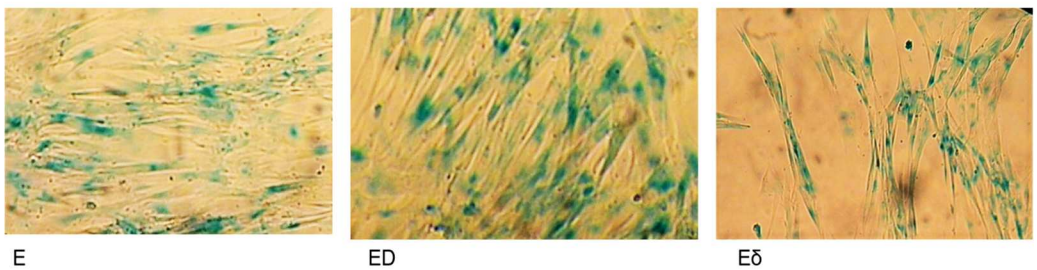

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◂**Fig. 3** Effect of γ- and δ-tocotrienols cotreatment on activity of SA-β-galactosidase under stress inducing premature senescence in MRC-5 human lung fibroblasts **a** P—cells treated with 100 μM hydrogen peroxide for 0.5 h **b** E–cells treated with 80 μM etoposide for 1 h. PD/ED—untreated control—cells treated with dimethyl sulfoxide 500 × diluted in the medium but not treated with tocotrienols. The values are means ± SD of three independent experiments ($n = 15$). Statistical significance was determined by the One-way ANOVA with the Bonferroni correction. P vs PD; PD vs Pγ; PD vs Pδ; E vs ED; ED vs. Eγ; ED vs Eδ; b-$p < 0.01$; c-$p < 0.001$ **c, d** PG–cells treated with 300 μM γ-tocotrienol; Ede/Pde–cells treated with 300 μM δ-tocotrienol; 100 × magnification

percentage of senescent cells does not change. These findings indicate that γ-and δ-tocotrienols do not act selectively against molecular processes which trigger senescence. γ-and δ-tocotrienols might induce cell death in cells growing in such a stress conditions as e.g. oxidative stress or DNA damage but do not affect healthy cells. On the other hand other studies have concluded that tocotrienols reversed cellular aging or improved cell viability [25, 26]. However, these studies were focused only on investigating the effects of tocotrienols on replicative senescence of cells growing in the medium without stress inductors such as hydrogen peroxide or etoposide. Moreover, we studied a different type of cells–MRC-5 cell line which was derived from lung fibroblasts, the other studies investigated myoblasts or dermal fibroblasts [25, 26].

We have also investigated expression of caspase-3 protein and apoptotic DNA fragmentation by the horizontal electrophoresis after cotreatment with γ- or δ-tocotrienols as we hypothesized that these treatments could lead to apoptosis. Caspase-3 is a key apoptotic protein common for intrinsic and extrinsic apoptotic pathways [27]. Malavolta et al. (2018) suggested that tocotrienols could lead to apoptosis in senescent cells since part of the metabolic and apoptotic pathways affected by tocotrienols in cancer cells overlap with those affected by quercetin which causes death of senescent cells by apoptosis [28, 29]. Administration of quercetin to senescent human umbilical vein endothelial cells led to the decreased expression of anti-apoptotic protein Bcl-2 and together with dasatinib (a drug used for treating cancer) quercetin was able to induce apoptosis in senescent preadipocytes [29]. Nevertheless, we did not detect the expression of caspase-3 or apoptotic DNA fragmentation after cotreatment with γ-tocotrienol or δ-tocotrienol. Although tocotrienols and quercetin affect the same part of metabolic and apoptotic pathways in cancer cells, their effect on senescent cells can be different. Moreover, quercetin used alone was not able to reduce viability of senescent preadipocytes, which indicates that the effect of quercetin depends on the cell type [29]. Similarly, the effect of tocotrienols on induction of apoptosis in senescent cells could depend on the cell type.

However, we found that cotreatment with γ-tocotrienol led to the increased level of LC-3 II protein in cells in which senescence was induced by hydrogen peroxide and cotreatment with δ-tocotrienol led to the increased level of LC-3 II protein in both models of senescence. LC-3 is an autophagic protein which exists in two forms LC-3 I and LC-3 II. LC-3 I is formed by postranslational modification. LC-3 II is formed by conjugation of LC-3 I to phosphatidylethanolamine which is localized on autophagosomes thus reflecting amounts of autophagosomal structures in cell [30, 31]. Increased level of LC-3 II protein occurs during some types of cell death and may be a sign of elevated autophagy as well as defective autophagy [14].

Furthermore, our results show that δ-tocotrienol is more effective than γ-tocotrienol since δ- tocotrienol of equal concentration as γ-tocotrienol has stronger effects on the decrease of cell viability/proliferation, the decrease of the total cell number and the increase of LC-3 II protein expression in our cell models of senescence. Similarly, Yam et al. have observed that δ-tocotrienol is the most effective anti-inflammatory agent compared to other tocotrienols [32] and Lim et al. have found out that δ-tocotrienol has a higher effectiveness in carrying out apoptosis in human lung adenocarcinoma A549 and glioblastoma U87MG cells compared to α-tocotrienol and γ-tocotrienol [33]. However, another study has reported that α-tocotrienol and γ-tocotrienol are more efficient than δ-tocotrienol in inhibiting proliferation of human cervical cancer cells [34]. Moreover, Jiang et al. have reported that γ-tocotrienol may inhibit cyclooxygenase-2 mediated production of prostaglandin E2 in IL-1β treated A549 cells most efficiently compared to other tocotrienols [35]. On the other hand, γ-tocotrienol as well as δ-tocotrienol both can effectively prevent Akt activation, reduce phospho-ERK, total ERK and phospho-mTOR protein levels and induce translocation of FOXO-3A from the cytoplasm to the nucleus in pancreatic cancer cells [36].

These observations suggest that effectiveness of tocotrienols depends on the type of cells and also on the type of molecular processes which are studied.

In conclusion, our present study suggests that γ- and δ-tocotrienols interfere with stress-induced premature senescence leading to the decreased viability of cells and increased expression of autophagic protein LC-3 II depending on the type of inductor of senescence and the type of tocotrienol used. According to our work δ-tocotrienol is more effective than γ-tocotrienol since δ-tocotrienol of equal concentration as γ-tocotrienol has stronger effects on the decrease of cell viability/proliferation, the decrease of total cell number and the increase of LC-3 II protein expression in our cell models of senescence.

Tab. 1 Effect of γ- or δ-tocotrienols cotreatment on activity of SA-β-galactosidase under stress inducing premature senescence in MRC-5 human lung fibroblasts

Cotreatment	Senecent cells % ± SD	All cells (%)	Statistical significance
100 μM Hydrogen peroxide/0.5 h	84.05 ± 5.45	100	
100 μM Hydrogen peroxide + DMSO 500x/0.5 h (PD control cells)	88.7 ± 3.56	100	*
100 μM Hydrogen peroxide + 300 μM γ-tocotrienol/0.5 h	79.12 ± 7.19	100	***
100 μM Hydrogen peroxide + 300 μM δ-tocotrienol/0.5 h	84.61 ± 4.99	100	*
80 μM Etoposide/1 h	90.36 ± 2.20	100	
80 μM Etoposide + DMSO 500x/1 h (ED control cells)	87.41 ± 2.9	100	*
80 μM Etoposide + 300 μM δ-tocotrienol/1 h	86.8 ± 4.56	100	*n*

The values are means ± SD of three independent experiments; $n = 15$; Statistical significance was determined by the One-way ANOVA with the Bonferroni correction. P vs PDMSO; PDMSO vs Pγ; PDMSO vs Pδ; E vs EDMSO; EDMSO vs Eδ

n nonsignificant

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

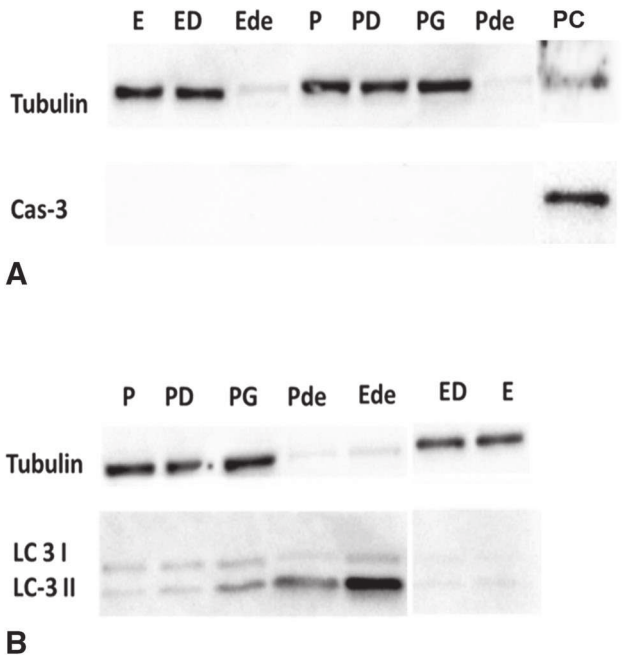

Fig. 4 Cas-3 protein level after cotreatment with γ- or δ-tocotrienols (**a**) and LC-3 protein level after cotreatment with γ-tocotrienol or δ-tocotrienol (**b**) E–cells treated with 80 μM etoposide for 1 h (10 μg); P–cells treated with 100 μM hydrogen peroxide for 0.5 h (10 μg); ED/PD—untreated control—cells treated with dimethyl sulfoxide 500 × diluted in the medium but not treated with tocotrienols (10 μg); PG–cells treated with 300 μM γ-tocotrienol Ede/Pde–cells treated with 300 μM δ-tocotrienol (5 μg); *PC* positive control, L1210 mouse lymphocytic leukemia cells treated by 6 μM cisplatin. The pictures represent two independent experiments

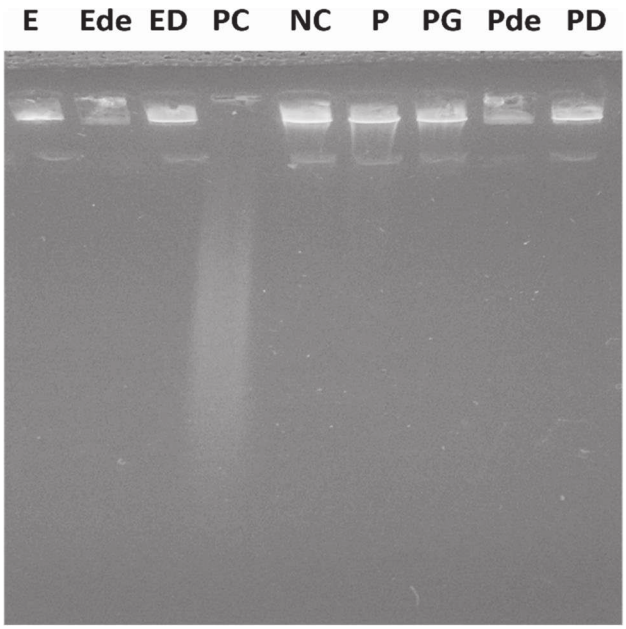

Fig. 5 Detection of apoptotic DNA fragmentation after cotreatment with γ- or δ-tocotrienols E—cells treated with 80 μM etoposide for 1 h; P–cells treated with 100 μM hydrogen peroxide for 0.5 h; ED/PD—untreated control—cells treated with dimethyl sulfoxide 500 × diluted in the medium but not treated with tocotrienols; PG – cells treated with 300 μM γ-tocotrienol; Ede/Pde–cells treated with 300 μM δ-tocotrienol; *NC* normal cells (without tocotrienols and dimethyl sulfoxide); *PC* positive control, L1210 mouse lymphocytic leukemia cells treated by 6 μM cisplatin. The picture represents two independent experiments

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Compliance with ethical standards

Conflict of interest None.

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Affiliations

Maria Janubova[1] · Jozef Hatok[2] · Katarina Konarikova[1] · Ingrid Zitnanova[1]

✉ Maria Janubova
maria.janubova@fmed.uniba.sk

[1] Faculty of Medicine, Institute of Medical Chemistry, Biochemistry and Clinical Biochemistry, Comenius University, Bratislava, Slovakia

[2] Jessenius Faculty of Medicine, Department of Medical Biochemistry, Comenius University, Bratislava, Martin, Slovakia