

RADIOPROTECTIVE ROLE OF α -TOCOTRIENOL AGAINST OXIDATIVE DAMAGE IN γ -IRRADIATED HUMAN BLOOD CULTURES

PAPEL RADIOPROTECTIVO DO α -TOCOTRIENOL CONTRA DANOS OXIDANTES NAS CULTURAS DE SANGUE HUMANO γ -IRRADIADAS

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ABSTRACT: Tocotrienols are members of the natural vitamin E family which is considered one of important fat soluble vitamins. The tocotrienols react with free radicals, which are the main cause of oxidation damage to cell membranes, without formation of other free radicals in the process. All natural forms of tocotrienols have the ability to regulate peroxidation reactions and to control free radicals production within the body. This study aimed to assess the antimutagenic and antioxidant ability of α -tocotrienol at a working dose (0.04 mg/ ml) through cytogenetic (Micronucleus test) study and biochemical analysis including Caspase -3, Superoxide dismutase (SOD), Catalase (CAT) activities and Nitric Oxide (NO) concentration in γ -irradiated human blood cultures. The treatment time was 72 hrs post-irradiation with gamma rays at dose of 3 Gy. Triple blood cultures for each blood sample were set up. Ionizing irradiation induces a significant increase in micronuclei (MNI) frequencies, and nucleoplasmic bridge (N bridge) accompanied by a significant rise in Caspase-3 activity and NO concentration. Furthermore, SOD and CAT activities showed significant decrease. α -tocotrienol treatment results into a decrease of MNI and N bridges numbers, enhancement of SOD and CAT activities and improvement of both NO and Caspase-3 levels, compared to irradiated cells which not treated with α -tocotrienol. The present results reveal the antimutagenic and the anti-oxidant effects of α -tocotrienol against γ - irradiation.

KEYWORDS: Vitamin E. γ -rays. Micronucleus test. Nitric oxide. Caspase-3.

INTRODUCTION

Vitamin E family consists of two subgroups; tocotrienols and tocopherols. Both of them have a chromanol nucleus, which is the site of antioxidant activities but differ in the tail region of the molecule (ATKINSON et al., 2008). Tocotrienols are novel components and naturally derived from several sources, including rice bran, palm, and annatto (MULLER et al., 2010).

Alpha, beta, gamma, and delta are among the isomers of tocotrienols. The potency of cancer treatment by these tocotrienols isomers is arranged in ascending order as following; beta, alpha, gamma then delta (AGGARWAL et al., 2010). The antioxidant efficiency of tocotrienols was evaluated as the ability of the compounds to inhibit lipid peroxidation, reactive oxygen species (ROS) production, and heat shock protein expression (PALOZZA et al., 2006).

Exposure to ionizing radiation became a part of our lives in different ways such as medical, environmental, and/or even accidental. Thus, accepting of the mechanisms by which radiation toxicity develops is crucial to statement acute and chronic health problems that occurs following ionizing radiation exposure. Direct exposure to

ionizing radiation-induced free radicals is totally recognized as significant contributors to early and late effects of ionizing radiation. This includes but is not limited to inflammation, cytotoxicity and genotoxicity (PATHAK et al., 2015). From that point of view using antioxidant and potent anti-inflammatory agent is mandatory request.

Many studies have performed extensive research on tocotrienols as a radiation countermeasure agent. ROS and reactive nitrogen species (RNS) are the primary source of radiation-induced damage, and tocotrienols (as potent antioxidants) are effective radioprotectors, supporting the hypothesis that “strong antioxidants make strong radio protectors” (GHOSH et al., 2009).

The dicentric chromosomes examination in human lymphocytes is a well-known method accomplished since long ago to estimate radiation exposure risk. The frequency of MNI is also commonly used as a cytogenetic biomarker. Another cytogenetic endpoint, cytokinesis-block micronucleus (MN) assay, is considered to be simple in terms of scoring criteria as a reliable and sensitive cytogenetic biomarker (SAMARTH et al., 2015).

Antioxidants suppress double strand breaks and chromosome aberrations in irradiated human lymphocytes (BRAND et al., 2015). The vitamin E isoforms tocotrienol are natural dietary antioxidant and a potent radioprotector, with the largest dose reduction factor among all the natural products (GHOSH et al. 2009). Many studies do not fully understand how tocotrienol protects against radiation damage but it may regulate apoptotic signals, cytokine production, cholesterol biosynthesis, or progenitor cell mobilization (PATHAK et al., 2016). Moreover, gene-expression profiles suggest that γ -tocotrienol affects a number of genes known to be important for the DNA damage response (BERBEE et al., 2012).

SOD and CAT enzymes are involved in oxidative stress and used to monitor the development and extent of oxidative stress damage (RENDIC AND GUENGERICH, 2012). In addition, NO is a highly diffusible regulator of several physiological processes. NO has been shown in numerous investigations to be involved in the cellular response to ionizing radiation (LEACH et al., 2002).

Caspases are a family of genes important for maintaining homeostasis through regulating cell death and inflammation. Deregulation of caspases underlies human diseases including cancer and inflammatory disorders, and major efforts to design better therapies for these diseases seek to understand how these enzymes work and how they can be controlled (MCLLWAIN et al., 2013). This study aimed to assess the antimutagenic and antioxidant ability of α -tocotrienol at a working dose (0.04 mg/ml) through cytogenetic (Micronucleus test) study and biochemical analysis including Caspase -3, Superoxide dismutase (SOD), Catalase (CAT) activities and Nitric Oxide (NO) concentration in γ -irradiated human blood cultures.

MATERIAL AND METHODS

Chemicals

The chemicals of the blood cultures were purchased from GIBCO-BRL, USA. Cytochalasin-B, heat-(BN) were assayed by blocking the cells at the inactivated foetal calf serum (FCS), D- α -tocotrienol and cytokinesis stage by the method of Fenech and solvents were purchased from Sigma/ Aldrich Chemical Co., St. Louis, USA.

Blood sampling

In the purpose of avoiding possible inter-individual variability in response to treatments, blood samples were obtained from equivalent five healthy volunteers (Men, average age 35 years and non-smokers) who offered an informed permission

for contribution in this study. Donors were chosen carefully according to the International Programme on Chemical Safety guidelines for the monitoring of genotoxic effects of carcinogens in humans (ALBERTINI et al., 2000). Venous blood were collected under sanitary conditions in heparinised vacutainer test tubes (V= 5 ml, Becton Dickinson, USA) containing lithium heparin to avoid blood coagulation.

Experimental design

For each donor sample, blood was divided into 5 groups (n=5): in each group 3 samples were processed. Experimental groups intended as following: 1- Control group: control blood, 2- Dimethyl sulfoxide (DMSO) group: blood treated with DMSO as vehicle, 3- α -tocotrienol group: blood samples treated with (0.04 mg/ml) of α -tocotrienol, 4- Irradiated group: blood samples exposed to 3Gy of γ -rays and 5- Irradiated and α -tocotrienol group: which represents blood treated with α -tocotrienol after irradiation.

Blood culture

To 0.5 ml of the whole blood, 5ml culture medium (RPMI-1640) supplemented with 20% FCS, 200 mM l-glutamine, penicillin 100 units/ ml and streptomycin 100 μ g/ ml were added in 15ml conical tubes. Phytohaemagglutinin-M with dose 0.2 ml was added to the culture to initiate cell division. Then, cells were incubated at 37°C.

Irradiation source

γ -rays were delivered to cell cultures through Canadian ¹³⁷Cs source belonging to the National Centre of Radiation Research and Technology, Atomic Energy Authority, EGYPT. The adjusted dose was 3 Gy. The dose rate was 0.42 Gy/ min. The samples were kept at 37°C after irradiation immediately till the treatment periods in the cultures with α -tocotrienol started.

Cytogenetic analysis

Cytokinesis-blocked micronucleus assay (CBMN)

The presence of MNi in a binucleated cells were assayed by blocking the cells at the cytokinesis stage by the method of Fenech and Morley (1985) and Fenech et al. (2003) and its modification in Fenech (2007). Blood culture was set as described previously. Cytochalasin-B (3 μ g/5ml culture) was added to the culture at 48h after the initiation. The cells were further incubated at 37°C for another 24h. At the harvest time, after centrifugation at 1500 rpm for 5min, cell pellets were treated with 5ml of mild hypotonic solution (0.1MKCl) for 3min, and a further 10min of

centrifugation at 800rpm. After another centrifugation, cells were washed once with the fixative solution (3:1 (v/v) methanol: acetic acid). Fixed cells were dropped gently on clean microscope slides, air-dried and stained with 10% Giemsa for 8min. In each group a total of 1500 BNC (500 from each experiment) were scored and the frequency of cells with one MN, two MNi and N bridge were recorded.

Biochemical analysis

Determination of SOD activity

In SOD activity determination Xanthine-xanthine oxidase was used to generate $O_2^{\bullet-}$ and nitroblue tetrazolium (NBT) decline was used as an indicator of $O_2^{\bullet-}$ production. SOD will compete with NBT for $O_2^{\bullet-}$; the percent inhibition of NBT reduction is a measure of the amount of existing SOD. SOD activity was measured in both 48 & 72 hrs cell culture supernatants. For each tube 0.5 of cacodylic buffer 0.1ml of triton X100, 0.25 ml of NBT and 1 ml of cell culture supernatants were mixed, then the mixture was incubated for 5 min at 37°C then start the reaction by adding 0.1 ml of pyrogallol, after 5 min add 0.3 ml stop solution was added to stop the reaction. 1.0 ml of distilled water was added instead of cell culture supernatant in blank. Finally at 540 nm optical density was measured spectrophotometrically. This quantitative method was carried on according to Sun et al., (1988).

Determination of Catalase activity

CAT activities were measured in cell culture supernatant, according to the method of Johansson and Borg (1988) based on the reaction of CAT enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. 400 μ l of cell culture supernatant, 50 μ l of methanol, 50 μ l of $KH_2PO_4/NaOH$ and 10 μ l hydrogen peroxide were mixed and incubated in 50 μ l of potassium hydroxide, then 100 μ l of purpled solution were added to the mixture and incubated again in water bath for 10 min at 20°C then 50 μ l of potassium periodate were added to the mixture then centrifuged at 9500 g for 10 min. The coloured compound developed from the reaction of purpled and formaldehyde was measured spectrophotometrically at 550 nm.

Measurement of NO

NO was measured according to the manufacturer instruction using the QuantiChrom™ Nitric Oxide Assay Kit (D2NO-100). This BioAssay Systems' Assay Kit was

designed to accurately measure NO production following reduction of nitrate to nitrite using improved Griess method. The absorbance was read at 540 nm.

Immunoblotting and Caspase-3 detection

Determination of caspases-3 immunoblot analysis was performed using a Nova Blot semi-dry blotter (LKB, Bromma, Sweden). Preparation of buffers, samples and transfer procedure were carried according to the method of Towbin et al. (1979).

Statistical analysis

Data obtained are represented as mean \pm standard error. Statistical analysis was carried out using the Statistical Package for Social Science (SPSS) software, significant differences among groups were evaluated using one-way analysis of variance (one-way ANOVA); least-significant difference was used for multi-group comparisons. *P* values ≤ 0.05 were considered as significant.

RESULTS

As shown in (Figure 1) the percentages of the BN with one, two and three MNi in human lymphocytes culture exposed to 3 Gy of γ -rays was significant, compared with control and α -tocotrienol groups. The results revealed significant improvements in all percentages of MNi after α -tocotrienol treatment although still significantly higher than that of control group.

On the other hand, data in (Figure 2) indicates that α -tocotrienol treatment significantly decrease the mean total aberrant cells but can't affect the mean N bridges caused by irradiation in significant way.

Figure 3 shows that SOD activity decreased after ionizing radiation exposure, α -tocotrienol enhanced SOD activity. (Figure 4) illustrates the same effect for CAT activity while, using α -tocotrienol after irradiation caused enhancement of its activity.

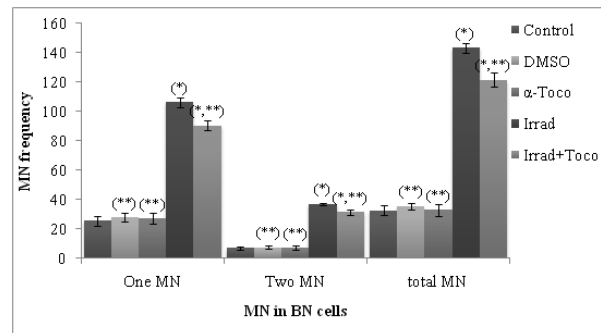


Figure 1. MN test in human blood culture (*In Vitro*) treated with α -tocotrienol after 3Gy γ -rays exposure. Statistical significance value ($P < 0.05$); * Significant when compared with control group; ** Significant when compared with radiation group; α -Toco: α -tocotrienol group; Irrad: Irradiation group; Irrad + Toco: Irradiated and α -tocotrienol group.

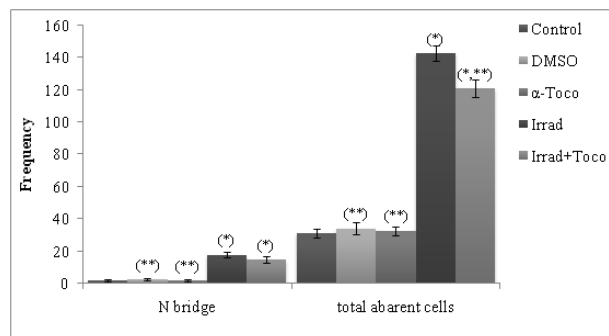


Figure 2. Frequencies of N- bridges and aberrant cells in human blood culture (*In Vitro*) treated with α -tocotrienol after 3Gy γ -rays exposure. Statistical significance value ($P < 0.05$); * Significant when compared with control group; ** Significant when compared with radiation group; α -Toco: α -tocotrienol group; Irrad: Irradiation group; Irrad + Toco: Irradiated and α -tocotrienol group.

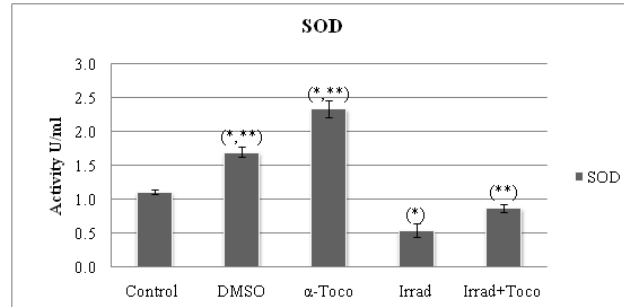


Figure 3. SOD activity of human blood culture treated with α -tocotrienol after 3Gy γ -rays exposure. Statistical significance value ($P < 0.05$); * Significant when compared with control group; ** Significant when compared with radiation group; α -Toco: α -tocotrienol group; Irrad: Irradiation group; Irrad + Toco: Irradiated and α -tocotrienol group.

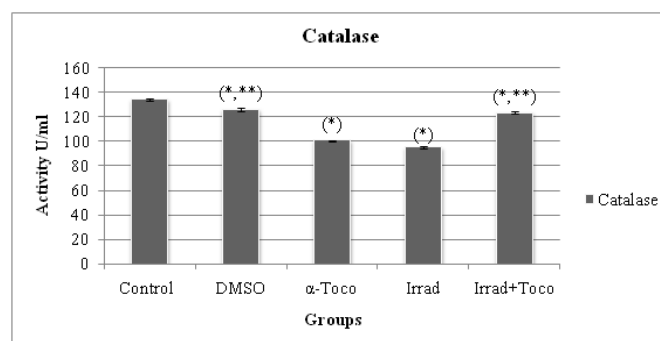


Figure 4. Catalase enzyme activity of human blood culture treated with α -tocotrienol after exposing to γ -radiation. Statistical significance value ($P < 0.05$); * Significant when compared with control group; ** Significant when compared with radiation group; α -Toco: α -tocotrienol group; Irrad: Irradiation group; Irrad + Toco: Irradiated and α -tocotrienol group.

Radiation exposure cause significant increase in NO concentration in culture media while α -tocotrienol treatment resulted in amelioration in its concentration as shown in (Figure 5).

Processing of pro caspase-3 (inactive form) into caspases-3 (active form) was examined by western immunoblotting. The results obtained in (Figure 6) revealed that α -tocotrienol group alone

showed mild caspase-3 activation compared with control group at each time interval. Caspase-3 was strongly activated 72 hrs post irradiation. Combination of both treatments (irradiation and α -tocotrienol) showed significant deactivation of caspase-3. It was clear that DMSO group recorded inactivation of caspases-3 processing.

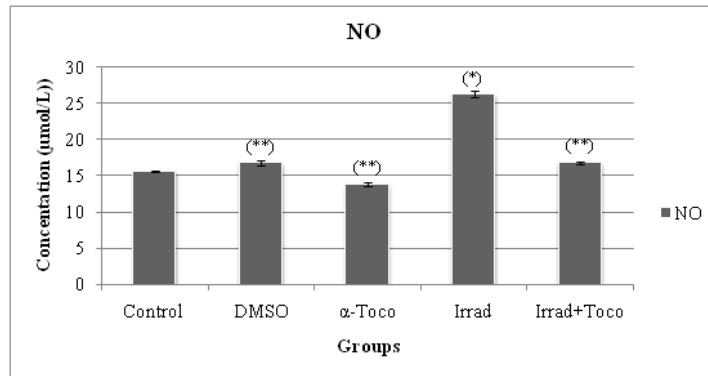


Figure 5. Nitric oxide concentration in human blood culture treated with α -tocotrienol after 3Gy γ -rays exposure. Statistical significance value ($P < 0.05$); * Significant when compared with control group; ** Significant when compared with radiation group; α -Toco: α -tocotrienol group; Irrad: Irradiation group; Irrad + Toco: Irradiated and α -tocotrienol group.

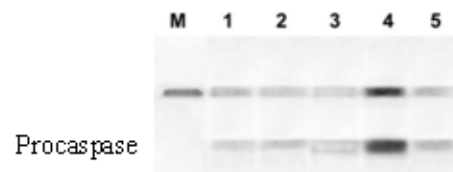


Figure 6. Caspase-3 cleavage in human lymphocytes treated with α -tocotrienol after γ -radiation exposure. M is marker. Lane 1: control group. Lane 2: DMSO treated group, lane 3: α -tocotrienol subjected group, lane 4: irradiated group, lane 5: Irradiated and α -tocotrienol group.

DISCUSSION

Ionizing radiation possesses sufficient energy to strip electrons out of atoms or molecules to create atoms or molecules with unpaired electrons called free radicals. At sufficiently high doses, ionizing radiation induces ionization events leading to damage to DNA, proteins, or membrane lipids, either directly or indirectly through the intracellular generation of ROS including superoxide anion, hydrogen peroxide, hydroxyl radicals, peroxide radicals, and other free radicals.

ROS which are generated by radiolysis of water with the direct action of radiation or indirectly through production of free radicals from other molecules causes DNA damages (SAMARTH et al., 2015). The present study indicated that α -tocotrienol treatments reduced the numbers of radiation-induced MNi, total aberrant cells and N bridges (Figures

1&2). These observations are in agreements with previous studies indicated that free radicals arising during radiation-exposure can lead to DNA damage. If overproduction of these radicals occurs, oxidative damage could lead to radiation-induced cytotoxicity; and MNi expression (HALLIWELL, 2002). So, determination of micronucleated cells and chromosome aberrations frequencies has a valuable role in monitoring populations exposed to natural or synthetic mutagenic agents (SILVA et al., 2016).

Khabour et al. (2013) suggested that various vitamin E isoforms, including gamma tocotrienol, protect against cytogenetic damage in humans. A commercially available, tocotrienol-rich vitamin E derivative decreases sister chromatid exchanges in elderly human subjects (CHIN et al., 2008). Thus, multiple vitamin E isoforms could be used to prevent radiation induced cytogenetic damage. It was found that gamma tocotrienol modulates as

many as 27 gene clusters in human umbilical vein endothelial cells involved in DNA damage (BERBEE et al., 2012).

Pathak et al. (2016), suggested several mechanisms to explain how gamma-tocotrienol detoxifies ROS. Gamma-tocotrienol may regulate mitochondrial activity, transcription factors, or endothelial cell surface receptors. The present data indicate that α -tocotrienol protects the lymphocytes from ROS mediated DNA damage (MN), and may do so in a number of ways. Miss-repaired DNA double strand break induce MN that are visible in lymphocytes. It was found that α -tocotrienol decreases MN and total aberrant cells in cultured human lymphocytes.

Recognition of radiation-induced ROS as the proximate cause of cellular death has fuelled the quest for identification of antioxidant molecules that could serve as potential radiation countermeasures (DUMONT et al., 2010). An obvious solution would be to stimulate the innate intracellular mediators of the antioxidant response (glutathione, thioredoxin, SOD, and CAT) that may be overwhelmed by the sudden burst of oxygen free radicals induced by radiation. Alternatively, extrinsic supplementation of these same intrinsic defense mechanisms can result in scavenging of free radicals before they damage critical cellular structures and functions. Lastly, analogs of such intrinsic scavengers administered after the radiation exposure can serve as reducing agents, or newer synthetic molecules that have the same scavenging properties can be custom-designed. Prominent among these strategies is the use of thiol compounds, polyphenols, SOD mimetics, and vitamin E analogs, all of which have been investigated as potential radiation countermeasures in recent years (WEISS; LANDAUER, 2009).

RNS are the main source of radiation-induced injuries, and tocotrienols have radio protector properties, supporting the hypothesis that "strong antioxidants make strong radio protectors" (GHOSH et al., 2009). After ionizing irradiation macrophages as being radioresistant, long survival, get activated, and produce large amounts of NO (GHOSH et al., 2008; JIANG et al., 2010). Nevertheless, amelioration of radiation damage is due to tocotrienol's antioxidant properties. The first site of injury by radiation to the human body is the bone marrow that produces blood. Delta- and α -tocotrienols induce an obvious stimulatory effect on hematopoietic tissue (SATYAMITRA et al., 2011). The results of the present study are in agreement with previous studies and suggest that α -tocotrienols, could be used as a powerful radio

protectors in nuclear risk areas, radiation workers and cancer radiotherapy patients.

Caspases family members are at the nexus of critical regulatory networks controlling cell death and inflammation. It is known that although caspase activity is critical for homeostasis of organisms, cells must take steps to protect themselves against unplanned caspase activation through complex systems required to turn inactive caspase zymogens into functional proteases. The long list of diseases associated with caspases tells us that the inappropriate activation of caspases and dysregulation of the cell death and inflammatory pathways they control has horrible consequences for human health. A growing body of research is providing us with ever increasing clarity about how these exciting proteases operate and how we might fight disease by manipulating their functions (BOATRIGT; SALVESEN, 2003).

Inhibition of caspase activity has had less striking therapeutic effects than has caspase activation. But, there are several instances in which, regardless of whether caspases have been definitively implicated in the pathological consequences of a disease, caspase inhibition has ameliorated the symptoms of several conditions caused by unsuitable apoptotic cell death. For example, because chronic hepatitis virus C infection is accompanied by detrimental hepatocyte apoptosis, a recent clinical trial examined the therapeutic potential of a caspase inhibitor (MANNES ET AL., 2010).

In the present study, caspases-3 activation was remarkably observed in irradiated group compared with irradiated then treated with α -tocotrienol revealing its ability of caspases-3 inhibition.

Tocotrienols radioprotective activities have been attributed to many explanations: uniform distribution in lipid bilayer due to its unsaturated aliphatic tail, which aids penetration into tissues, recycling efficiency (SERBINOVA; PACKER 1994) rate of cellular uptake, which is 70 times greater for tocotrienol than for α -tocopherol (SATIO et al., 2004).

Although many studies reported that the antioxidant effect of tocotrienols is greater than tocopherols, however, others studies reported no differences in the antioxidant potential of tocopherols and tocotrienols (MÜLLER et al., 2010). The explanation might be that the reducing and scavenging activity of tocols undoubtedly depends on the experimental design and the circumstances under which assays are performed.

All the tocotrienols and tocopherol isomers have antioxidant activity due to the ability to donate a hydrogen atom from the hydroxyl group on the chromanol ring, to free radicals and ROS. This process inactivates the free radical by donating a single unpaired electron to the radical (FU et al., 2014). The antioxidant effectiveness of tocotrienols is due to their ability to inhibit lipid peroxidation and ROS production (PALOZZA et al., 2006).

CONCLUSION

The anticlastogenic, antioxidant and antiapoptotic actions of α -tocotrienol can be attributed mainly to easy distribution between cells, high rate of cellular reuptake and free radicals

scavenging capacity. α -tocotrienol also display a great capability in inhibiting apoptosis through controlling initiator caspases inactivation

Conflict of interest

The authors declare that: there is no conflict of interest. Our research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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RESUMO: Os tocotrienóis são membros da família natural de vitamina E, que é considerada uma das vitaminas importantes solúveis em gordura. Os tocotrienóis reagem com os radicais livres, que são a principal causa do dano de oxidação das membranas celulares, sem formação de outros radicais livres no processo. Todas as formas naturais de tocotrienóis têm a capacidade de regular as reações de peroxidação e controlar a produção de radicais livres dentro do corpo. Este estudo teve como objetivo avaliar a capacidade antimutagênica e antioxidante do α -tocotrienol em uma dose de trabalho (0,04 mg/ml) através de estudo citogenético (Teste dos Micronúcleos) e análise bioquímica incluindo atividades de Caspase 3, Superóxido Dismutase (SOD), Catalase (CAT) e concentração de óxido nítrico (NO) em culturas sanguíneas humanas irradiadas com γ . O tempo de tratamento foi de 72 horas pós-irradiação com raios gama na dose de 3 Gy. Foram estabelecidas culturas de sangue triplas para cada amostra de sangue. A irradiação ionizante induz um aumento significativo nas frequências de micronúcleos (MNI) e ponte nucleoplasmática (ponte N), acompanhada por um aumento significativo da atividade da Caspase 3 e da concentração de NO. Além disso, as atividades de SOD e CAT mostraram queda significativa. O tratamento com α -tocotrienol resulta em uma diminuição do número de pontes MNI e N, aumento das atividades de SOD e CAT e melhora dos níveis de NO e Caspase 3, em comparação com células irradiadas que não foram tratadas com α -tocotrienol. Os presentes resultados revelam os efeitos antimutagênicos e antioxidantes do α -tocotrienol contra a irradiação γ .

PALAVRAS-CHAVE: Vitamina E; Raios- γ ; Teste dos micronúcleos; Óxido nítrico; Caspase 3.

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