Abstract

Objective: The free radical theory of aging (FRTA) suggests that free radicals are the leading cause of deteriorating physiologic function during senescence. Free radicals attack cellular structures or molecules such as DNA resulting in various modifications to the DNA structures. Accumulation of unrepaired DNA contributes to a variety of disorders associated with the aging process.

Methods: A randomized, double-blinded placebo-controlled study was undertaken to evaluate the effect of Tri E® Tocotrienol on DNA damage. Sixty four subjects 37–78 y old completed the study. A daily dose of 160 mg of Tri E® Tocotrienol was given for 6 months. Blood samples were analyzed for DNA damage using comet assay, frequency of sister chromatid exchange (SCE), and chromosome 4 aberrations.

Results: Results showed a significant reduction in DNA damage as measured by comet assay after 3 mo (P < 0.01) and remained low at 6 mo (P < 0.01). The frequency of SCE was also reduced after 6 mo of supplementation (P < 0.05), albeit more markedly in the >50 y-old group (P < 0.01) whereas urinary 8-hydroxy-2’-deoxyguanosine (8-OHdG) levels were significantly reduced (P < 0.05). A strong positive correlation was observed between SCE with age, whereas weak positive correlations were observed in DNA damage and 8-OHdG, which were reduced with supplementation. However, no translocation or a stable insertion was observed in chromosome 4.

Conclusion: Tri E® Tocotrienol supplementation may be beneficial by reducing DNA damage as indicated by a reduction in DNA damage, SCE frequency, and urinary 8-OHdG. © 2008 Elsevier Inc. All rights reserved.

Keywords: DNA damage; Sister chromatid exchange; Chromosome 4; 8-Hydroxy-2’-deoxyguanosine

Introduction

Reactive oxygen species (ROS) are formed continuously in living cells as a consequence of cellular metabolic and biochemical reactions and its rate of formation may be influenced by exogenous factors [1]. High levels of these radicals cause damage to sensitive biological structures such as DNA, lipids, and proteins. As a result, the cell has an elaborate system to maintain a proper balance between the levels of free radicals and antioxidants to ensure the integrity of cellular components [2]. DNA is considered to be the most sensitive cellular target because of the potential for accumulative mutations. It is thought that cumulative unsuccessful removal of DNA damage may give rise to genetic changes that can subsequently lead to numerous
age-related biological disorders by disrupting cellular homeostasis [1]. It has long been postulated that supplementation with dietary antioxidant can alleviate the redox imbalance and thereby protect against the deteriorating effects of oxidative stress, progression of degenerative diseases, and aging.

Vitamin E is considered to be the most potent lipid-soluble antioxidant and has been the subject of an increasing number of studies for the past few decades. Apart from its antioxidant property, vitamin E has been reported to have other biological functions such as enhancing immune response [3], modulating DNA repair systems [4], and in signal transduction pathways [5]. The current formulation of vitamin E consists primarily of α-tocopherol but recent research has suggested that tocotrienol, the lesser known form of vitamin E, appears superior regarding its antioxidant properties [6] and possesses unique biological functions unrelated to antioxidant activity not shared by tocopherol [7]. Even among the tocopherols, particular importance is placed on the other isomers because supplementation with large doses of α-tocopherol alone has been reported to deplete the availability of γ-tocopherol, thus denying the benefits of γ-tocopherol that are not shared by α-tocopherol [8]. Therefore, it has been suggested that the full benefits of vitamin E are better achieved by supplementation with the full spectrum of vitamin E isomers.

Conflicting results regarding the effects of vitamin E supplementation in reducing levels of free radical damage have been reported from human, randomized, controlled trials. This was most likely due to individual response differences as described by Pryor [9]. Epidemiologic evidence has indicated that high plasma concentrations of vitamin E are associated with a lower risk of cardiovascular disease [9–11] and certain types of cancer [12,13].

Tri E® Tocotrienol is a commercially available tocotrienol-rich vitamin E derived from palm oil. It is comprised of a combination of α-, β-, γ-, and δ-tocotrienol and α-tocopherol. The total tocotrienols isomer ratio compared with tocopherol is approximately 74%:26%.

The aim of the study was to determine the chromosomal stability resulting from Tri E® Tocotrienol supplementation by means of the alkaline comet assay, sister chromatid exchange (SCE) test, and fluorescent in situ hybridization application of chromosome 4. In addition, 8-hydroxy-2'-deoxyguanosine (8-OHdG), a well-established marker of oxidative stress, was determined in urine.

Materials and methods

Subjects

The study was approved by the research and ethics committee of the Medical Faculty, Universiti Kebangsaan Malaysia. Subjects enrolling in the study were recruited from among individuals who had been screened previously for population studies. All participants were normal healthy individuals, were at least 35 y of age, were informed, and consented to participate in the study. They were non-smokers, without any significant clinical diseases, not pregnant, and not taking any other medication, alcohol, or supplements.

Experimental study

A randomized, double-blinded, placebo-controlled study was carried out to determine the effect of Tri E® Tocotrienol on DNA damage. Tri E® Tocotrienol was supplied by Golden Hope Bioganic (Kuala Langat, Selangor, Malaysia) and consisted of approximately 74% tocotrienols and 26% tocopherol. Sixty-four subjects were studied for extent of DNA damage, frequency of SCEs and chromosome 4 aberrations, and the amount of 8-OHdG in whole blood leukocytes, lymphocytes, and urine, respectively. Subjects were randomly assigned to one of two groups in which 32 received Tri E® capsule (160 mg/d) supplementation and the other 32 received placebo. Subjects were requested to consume the Tri E® after dinner to ensure proper absorption [14,15]. Fasting blood (15 mL) and urine samples were taken from each subject before supplementation and at 3 and 6 mo of supplementation. Blood samples were collected into heparinized blood collection tubes and investigations done using fresh blood, and urine samples were aliquoted and stored at −80°C until analysis. Data collection was performed by questionnaires that included information on age, medical history, ethnicity, religion, marital status, occupation, socioeconomic status, physical activity, and diet. Participants were also required to undergo a full physical examination before enrollment.

Alkaline single-cell gel electrophoresis (comet assay)

The comet assay was performed as described by Singh et al. [16] with slight modifications. Briefly, fully frosted microscope slides were precoated with a layer of 0.6% normal melting point agarose at about 50°C. These were covered with coverslips to flatten out the molten agarose layer and chilled to enhance gelling of the agarose. The subsequent procedures were performed under dim light to prevent further DNA damage. Blood samples were suspended in 0.6% low melting point agarose in phosphate buffered saline, pH 7.4. After removing the coverslips from the slides, the cell suspensions were pipetted onto the first agarose layer at about 37°C and coverslips were applied once again. The low melting point agarose was allowed to solidify on an ice-cold flat tray. After removal of the coverslips, the slides were immersed into ice-cold lysing solution (2.5 M NaCl, 100 mM ethylene-diaminetetra-acetic acid, 10 mM Tris at pH 10, 1% Triton X-100, 1% sodium N-lauroyl sarcocinate, 10% dimethylsulfoxide) at 4°C for 1 h to remove cellular protein. Slides were then placed horizontally in an electrophoresis tank containing 0.3 M NaOH and 1 mM ethylene-diaminetetra-acetic acid at 4°C
for 20 min to allow DNA unwinding. Electrophoresis was performed at 25 V and 300 mA for 20 min. The slides were then washed three times for 5 min each with a neutralizing solution (0.4 M Tris, pH 7.5) before staining with 20 μL/mL of ethidium bromide. Coverslips were placed on the top and the slides were placed in a humidified container overnight.

Visual analyses of DNA damage were carried out in accordance with the protocols of Kobayashi et al. [17]. Slides were examined at 200× magnification using a fluorescence microscope. Five hundred randomly selected non-overlapping cells on each slide were analyzed and assigned grades on an arbitrary scale of 0 to 4 based on perceived comet tail length migration and relative proportion of DNA in the comet tail (0, no damage; 1, low level damage; 2, mild damage; 3, high level damage; 4, extensive damage) as shown in Figure 1. For final analysis, a total damage score of each slide was calculated according to the method as described by Heaton et al. [2]. Using this formula, the number of cells assigned to each grade of damage was multiplied by the numeric value of the grade and the sum of all grades provides a minimum possible score of 0, corresponding to 500 cells at grade 0, whereas a maximum possible score of 2000, corresponding to 500 cells at grade 4.

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\text{total DNA damage score} = 0 \times \text{(grade 0 cells)} + 1 \times \text{(grade 1 cells)} + 2 \times \text{(grade 2 cells)} + 3 \times \text{(grade 3 cells)} + 4 \times \text{(grade 4 cells)}
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SCE assay

Heparinized blood samples were coded before culturing. Two parallel cultures were set up for each subject, with 1 mL of whole blood added to 10 mL of RPMI-1640 culture medium supplemented with l-glutamine, 20% fetal calf serum, 2.5% phytohemagglutinin-M form, and 100 IU/mL of antibiotic (penicillin and streptomycin). The blood cultures were grown in a constant humidified atmosphere with 5% CO₂ at temperature of 37°C. At 24 h of culture, 0.1 mL of 0.01 M 5-bromodeoxyuridine was added into the culture flasks and the cells were incubated for an additional period of 48 h in the dark. One hour before harvesting (71 h), 0.1 mL of colchicine (0.1 µg/mL) was added to arrest cell growth at metaphase. After completion of 72 h of incubation, the cells were treated with a hypotonic solution (0.075 M KCl) and fixed in Carnoy’s fixative (methanol-glacial acetic acid, 3:1). The fixed cells were spread on clean slides. Differential staining of bromodeoxyuridine-substituted sister chromatids was obtained by the fluorescent plus Giemsa method of staining as described by Perry and Wolf [18], in which the slides were incubated with Hoechst 33258 (0.5 µg/mL) solution for 30 min, rinsed, and dried. The slides were then exposed to ultraviolet light in the presence of 2× standard saline citrate (SSC) solution for 2.5 h. The slides were again rinsed, dried, and incubated in 2× SSC solution at 60°C for 30 min. The slides were stained with 5% Giemsa stain and scored under the microscope. Fifty metaphases were scored blindly in random fashion from each sample for SCE. Only well-differentiated second-division metaphases were accepted for scoring [19]. The SCEs were counted and expressed as the number of SCEs per cell.

Fluorescent in situ hybridization assay

Blood cultures were established and fixed in Carnoy’s fixative as described above without the addition of bromodeoxyuridine. The cell samples were spotted onto clean microscopic slides and aged for 3 d at 56°C. Chromosome 4 was investigated using whole chromosome painting probe specific to chromosome 4 (whole chromosome painting chromosome 4 labeled with Spectrum Green, Cytocell

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Fig. 1. Representation comet images, with cell grades 0–1 based on perceived comet tail length migration and relative proportion of DNA in the comet tail (0, no damage; 4, extensive damage).
Technologies Ltd., Cambridge, UK) and blue stain (4,6-diamino-2-phenyl-indol, DAPI) to counterstain the remainder of the DNA. Briefly, the slides were immersed in 2× SSC, pH 7.0, and dehydrated in a cold ethanol series (70%, 95%, and 100%) for 2 min each, air dried, and covered with 10 μL of DNA probe. The slides were placed onto a 75°C hotplate and denatured for 2 min. Overnight hybridization was done in a humid, lightproof container at 37°C. The slides were then washed in 0.4× SSC/0.3% NP-40 at 72°C for 2 min and 2× SSC/0.1% NP-40 at room temperature for 30 s, followed by counterstaining with 10 μL of DAPI antifade, and covered and the color was allowed to develop in the dark for 10 min. Evaluation and counting of chromosomal aberrations were carried out using an Olympus BX40 reflected fluorescence microscope (Olympus Corp., Shibuya-ku, Tokyo, Japan) at 100× magnification and appropriate absorption and excitation filters (Olympus). All analyses of metaphase signals were performed on slide areas assessed for good cell morphology and complete, non-overlapping cells. At least 100 metaphases of chromosome were analyzed and translocations (reciprocal, terminal, and interstitial) involving the painted chromosomes were scored. Image acquisition was performed using a CCD camera and Cytovision software (Applied Imaging Corp. Santa Clara, CA, USA).

Urinary 8-OHdG assay

Urine samples were thawed and centrifuged at 1500 g for 5 min and the supernatant used for analysis. Urinary 8-OHdG was measured using an enzyme-linked immunosorbent assay kit (Bioxytech 8-OHdG EIA kit, OXIS International Inc., Foster City, CA, USA) according to the manufacturer’s instructions. Each sample was assayed in duplicate and the results were expressed as a ratio of 8-OHdG to creatinine.

Statistical analysis

Statistical analysis was performed using a mixed-model analysis of variance and the associations of all parameters measured with age were assessed by Pearson’s correlation coefficient using SPSS 11.5 (SPSS, Inc., Chicago, IL, USA). Mauchly’s test of sphericity was used to assess the homogeneity of variance and Dunnett’s test was performed to compare the means of the treatment group to the means of the control group. Bonferroni’s adjustment was applied to control the inflation of type I error across multiple tests for statistical significance evaluation. All data are presented as mean ± standard error of the mean. Significance was set at P < 0.05. PS 2.1.31 (Vanderbilt University Medical Center, Nashville, TN, USA) was used to determine the power of the study in which the probability of rejecting the null hypothesis with type I error was set at α = 0.05, given the specified sample size n = 32, a standard deviation σ = 0.65, and when the true difference in population means was δ = 0.66. The statistical power obtained from our approach was 0.9995.

Results

The extent of DNA damage as measured by the comet assay was attenuated by vitamin E supplementation as shown in Figure 2a. A lower basal level of DNA damage was recorded after 3 mo of supplementation and remained low at 6 mo compared with the level at the starting point of supplementation, whereas no changes were seen in subjects receiving placebo capsules. Analysis of DNA damage when the groups were further divided into two age groups comprised of a younger group (35–49 y old) and an older group (>50 y old; Fig. 2b) showed a different response. At baseline, total DNA damage was higher in older subjects in the placebo and Tri E® Tocotrienol supplemented groups compared with their younger counterparts (P < 0.05). Upon supplementation, the older subjects showed the greatest reduction in total DNA damage after 3 and 6 mo (P < 0.01), whereas in the younger group a lower level of DNA damage...
was observed only after 6 mo of Tri E® Tocotrienol supplementation ($P < 0.05$). The association of DNA damage with advancing age revealed a weak positive correlation (Fig. 3a) that grew weaker with supplementation (Fig. 3b).

Sister chromatid exchanges were analyzed from second-division metaphases using a staining method based on sister chromatid differentiation by bromodeoxyuridine labeling [20,21]. The appearance of the metaphase chromosome with these exchanges is shown in Figure 4. After quantification, the frequency of SCEs showed a relatively lower count in the Tri E® Tocotrienol supplemented group (Fig. 5a) after 6 mo ($P < 0.05$). Further analysis in which subjects were divided into two age groups revealed that only the older adults showed a decline in SCE count (Fig. 5b) after receiving 6 mo of Tri E® Tocotrienol supplementation ($P < 0.01$). Statistical covariation testing indicated a strong relation between the frequency of SCEs with age ($P < 0.01$) at baseline (Fig. 6a). Upon supplementation, the correlation grew weaker, indicating an improve-
ment in chromosomal stability in older adults (Fig. 6b). No changes were found in the placebo-receiving group (Fig. 4b). The range of SCEs per cell obtained is in good agreement with values reported by other researchers [22,23].

Fluorescent in situ hybridization is a molecular cytogenetic technique that allows the detection of chromosomal translocation of a particular chromosome in DNA. In our study, no translocations were seen in chromosome 4. All samples were normal as shown by fluorescent signals on the whole length of both homologous chromosomes 4, whereas the other remaining chromosomes were stained in blue by DAPI (Fig. 7).

Urinary excretion of 8-OHdG showed no significant changes, although there was a trend toward a decline in its level after supplementation (Fig. 8a) when the subjects were grouped together. Further analysis revealed that only the group of older adults (>50 y) showed a decrease in urinary 8-OHdG after 6 mo of Tri E® Tocotrienol supplementation (P < 0.05; Fig. 8b), whereas no changes were observed after 3 mo of supplementation. In the placebo group, the

Fig. 6. (a) Correlation between SCEs and age at baseline (0 mo). The placebo and Tri E® Tocotrienol groups showed a strong positive relation between the frequencies of SCEs with advancing age (placebo: r = 0.90, P < 0.01; Tri E® Tocotrienol: r = 0.72, P < 0.01). (b) Correlation between SCEs and age with Tri E® Tocotrienol supplementation for 6 mo. Tri E® Tocotrienol supplementation weakened the association after 3 and 6 mo (0 mo: r = 0.72, P < 0.01; 3 mo: r = 0.37, P = 0.09; 6 mo: r = 0.30, P = 0.18). SCE, sister chromatid exchanges.

Fig. 7. Chromosome 4 pair painted with the whole chromosome paint probe (fluorescent green) using fluorescent in situ hybridization.

Fig. 8. (a) Level of urinary 8-OHdG excretion/creatinine. Data expressed as mean ± SEM. No significant changes were observed in the placebo and Tri E® Tocotrienol supplemented groups. (b) Level of urinary 8-OHdG excretion/creatinine. Tri E® Tocotrienol supplementation reduced urinary 8-OHdG excretion after 6 mo (P < 0.05) in the older (>50 y old) group. Data expressed as mean ± SEM. *Significant level at P < 0.05. 8-OHdG, 8-hydroxy-2'-deoxyguanosine.
level of urinary 8-OHdG remained unchanged throughout the 6 mo of supplementation. Weak correlations were observed between urinary 8-OHdG and age in both groups (Fig. 9a). Supplementation with Tri E® Tocotrienol did not result in any statistically significant further changes to the level of urinary 8-OHdG with age (Fig. 9b) despite demonstrating a declining trend with increasing age with supplementation.

Discussion

DNA damage was significantly reduced after supplementation as shown by the comet and SCE assays, suggesting a protection by Tri E® Tocotrienol supplementation. Reduction in DNA damage as measured by the comet assay was observed as soon as 3 mo of supplementation and remained low with continued supplementation, whereas with SCE assay the reduction was observed in the older group after 6 mo of supplementation. Given a steady production of ROS in cells, single-stranded DNA breaks can be easily induced in leukocytes. Peripheral blood leukocytes comprise a non-cycling population and therefore it is possible that DNA damage detected by the comet assay were damages that had occurred in G0 phase cells. The comet test allowed the detection of single-strand breaks (SSBs) and double-strand breaks (DSBs) in addition to alkaline labile sites in all leukocytes [2]. The test is of biologically significance because it is a relative indication of the number of DNA strand breaks within the cell.

In contrast, SCEs are interchanges of DNA replication products between sister chromatids at apparently homologous loci [24,25]. They are suggested to represent homologous recombination repair of DNA DSBs [22]. SCEs were induced by long-lived DNA damage [26] that had occurred in T lymphocytes. Even though peripheral lymphocytes are normally non-cycling, they can be stimulated to enter the cell cycle by in vitro culturing with a mitogen such as phytohemagglutinin. SCEs were assessed in the second-metaphase lymphocytes, after the DNA repair process operation in G1 phase [27]. Therefore, damage must be present in S phase to be detected. Furthermore, SCE was evaluated only in the T lymphocytes [28], whereas the comet assay revealed DNA damage induced in all leukocytes [2], which may provide a more sensitive measurement of DNA damage as indicated by our results. Not only are the types of DNA damage measured in the comet assay and SCE different (with the comet assay being able to detect more types of DNA breaks), but also the time point in the cell cycle when these damages occurred were assessed.

The SSBs are produced during a normal cell cycle and are relatively harmless lesions [26] when present at a normal level that can be managed by the DNA repair system. However, abundant production of single-strand lesions can be converted to DSBs during the S phase of a cell cycle [27] under normal conditions and ROS are thought to increase SSBs.

When lesions occur, cell cycle checkpoints are activated in response to DNA damage to allow time for repair of induced DNA damage before its conversion into irreparable genetic alterations or induction of apoptotic cell death [27]. The most abundant spontaneous single-strand lesions are SSBs and the majority is repaired before DNA replication, but at least 1% escape repair [27]. These unrepaired lesions are encountered by replication forks and result in conversion to DSBs in one of the sister chromatids [24]. SSBs are repaired more efficiently than DSBs. Endogenous DSBs are processed by homologous recombination repair mechanisms, a relatively error-free mechanism for the repair of DSBs. Errors in repair may contribute substantially to the
formation of recombinagenic lesion upon DNA replication and this transformation of lesions is often related to carcinogenesis [26].

Age is an important contributing factor in describing DNA stability and compounds that alter ROS production such as antioxidant vitamins may also alter the extent of DNA damage. Data obtained at baseline suggested that DNA instability is likely to be age dependent as shown by the SCE assay, which increased significantly with age. SCE represents a spontaneous chromosomal response to DNA damage at the time of DNA replication [29]. The increase in chromosomal changes with age as reflected by the higher basal level of SCEs in older adults is associated with an accumulation of DNA damage with age and has been reported elsewhere [30]. This is in agreement with one of the predictions of the theories of aging involving DNA integrity in which cells from older individuals are expected to exhibit a greater amount of damaged DNA, possibly accompanied by a decreased efficiency in overall DNA repair capacity [30,31].

The effect of Tri E® Tocotrienol is more obvious in older age, possibly reflecting a greater need for supplementation or a greater profound effect due to the larger amount of damage present. Nutritional deficiency is thought to contribute to the decline in cellular immune function particularly among elderly men and women [32] beginning at approximately 50 y of age [33]. The decline has been associated with greater susceptibility of the elderly to many age-related diseases resulting in increased morbidity and mortality from such pathologic illnesses [34]. In the present study, we found that older individuals are at risk of having higher levels of DNA damage. Data from an animal model study have shown that the level of DNA lesions in elderly rats was twice the number in adolescent rats [35]. Further, with DNA repair capacity decreasing with advancing age [30,31], the possibility of age-related accumulation of DNA damage occurring is greater. Thus, it is proposed that older adults have a greater requirement for dietary antioxidant supplementation because they exhibit a higher level of DNA damage as shown in this study.

Tri E® Tocotrienol is a commercially available vitamin E supplement consisting of naturally occurring tocopherols and tocotrienols. The antioxidant effects of these compounds have been well documented but very few studies have investigated the effect of a mixture of vitamin E isomers particularly on DNA damage in older healthy humans. The protective effect of Tri E® Tocotrienol on DNA damage shown in our results is probably due to the inhibition of DNA damage formation by its antioxidant function and by activation of signaling pathways. Given that SCEs are manifestation of DSBs repair processes, the lower frequency of this biomarker observed in the Tri E® supplemented group could be explained by lower formation of DSBs, thus resulting in a decrease of DSB repair. Assuming that the rate of DNA repair activity remained consistent throughout the intervention, a lower formation of damage could result in a reduction of the need for repair to be performed. However, this theory does not necessarily apply to other types of DNA breaks and damage present. Another possible explanation is that it could be due to an increase in the rate of repair of damaged DNA. Mediation of DNA damage levels through the modulation of the DNA repair system through alteration of repair enzymes would result in an increased rate of removal of damaged DNA [4]. Tocotrienols have been suggested to act as a signaling molecule that modulates gene expression [36] and thus may be involved in increasing repair enzyme activities. However, further studies are required to elucidate the mechanism of action of tocotrienols in signal transduction.

Fluorescent in situ hybridization enables the evaluation of chromosome aberrations on a cell-by-cell basis. Chromosome 4 was selected for study on the basis of it having been associated with or linked to longevity [37]. No translocation was observed in this study, which could be attributed to the small sample. Translocation events involving very small chromosome parts of the short and/or long arms of chromosome 4 could have escaped unseen because whole chromosome paint was used in this study. Thus, only large translocations involving the painted chromosome are likely to be detected. Further investigations with a locus-specific probe may be required to study the chromosomal structure alterations in greater detail. The presence of translocations involving chromosome 4 has been reported to be associated with carcinogenesis [38–41] and genetic disorders [42,43].

Urinary excretion of 8-OHdG reflected the average rate of oxidative DNA damage in the whole body [44]. However, a decrease in urinary excretion of oxidized nucleotides may reflect lower levels of DNA oxidation or augmented enzyme repairing ability and does not reflect the steady state of unrepaired DNA damage level [45]. A decrease in urinary 8-OHdG observed in this study in the older supplemented subjects is in accordance with the comet and SCE results. The lower level of urinary 8-OHdG noted after the supplementation period could be attributed to lower levels of oxidized guanine in DNA at the intervention time point and thus reduced repair and formation of oxidative DNA modification product as shown in our study.

Conclusion

The results obtained suggested that supplementation with palm oil Tri E® Tocotrienol reduced the level of DNA damage in healthy subjects, with a more pronounced effect observed in older adults. These observations may indicate a possible relation between the molecular mechanisms involved in the formation and repair of DNA breaks with Tri E® Tocotrienol supplementation.
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