Gamma-Tocotrienol Pretreatment Reduces DNA Damage in Lymphocytes of Normal Subjects and Patients with Down’s Syndrome

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Abstract
The degeneration process associated with Down syndrome (DS) has been partly attributed to the excess hydrogen peroxide formation as a result of excess expression of SOD gene. The increased production of hydroxyl radicals result in damage to macromolecules including the DNA. In this study we evaluated the effect of gamma-tocotrienol (GTT), a subfamily of vitamin E, on DNA damage as measured by the single gel electrophoresis (otherwise known as the comet assay) in lymphocytes of normal and DS subjects. We also studied whether gamma-tocotrienol can reduce DNA damage when the DNA repair phase of the cell cycle is inhibited with caffeine. Blood samples were taken from 11 normal and 8 DS children aged 1-7 years old. The lymphocytes were harvested and then cultured in RPMI folate free medium for 72 h before treatment with 20 uM GTT or 5 mM caffeine. For the study on gamma-tocotrienol pretreatment on caffeine treated cells, the lymphocytes were incubated with GTT for 2 h before the addition of caffeine into the culture medium. Results showed that GTT significantly reduced basal DNA damage as well as when DNA repair was inhibited in lymphocytes taken from DS and normal subjects suggesting a potential role of GTT in the treatment and management of DS patients.

Keywords: comet assay, DNA damage, Down’s syndrome, γ-tocotrienol

Introduction
Down’s syndrome (DS) is one of the most common human autosomal abnormality. DS patients exhibit several major phenotypic features such as mental retardation, hypotonia, short stature, immunological and heart abnormalities and onset of neurochemical defects of Alzheimer’s disease after the 3rd or 4th decade of life (1). The syndrome results from inheriting three copies of chromosome 21 (trisomy 21) which is the location of the gene encoding the enzyme Cu/Zn superoxide dismutase (SOD1) (2). SOD1 catalyses the formation of hydrogen peroxide from superoxide radicals (3, 4). Normally the hydrogen peroxide (H₂O₂) produced is converted to water during reactions catalysed by glutathione peroxidase (GPx) and catalase (4). In DS, the altered ratio of SOD1 to GPx and catalase activity is believed to lead to H₂O₂ accumulation and increased oxidative stress (5). Increased levels of H₂O₂ via the Fenton reaction, can lead to the production of hydroxyl radicals and resultant damage to macromolecules. In this way, senescent changes is brought about through damage to important organelles such as DNA (6).

Indeed antioxidants such as vitamin E (γ-tocopherol) has been shown to protect against chromosomal damage (11).

In the present study we investigated the effect of another subfamily of vitamin E ie γ-tocotrienol which differs structurally from γ-tocopherol by possessing three double bonds in their isoprenoid side chains (12). Many studies have shown that γ-tocotrienol is a more potent antioxidant than γ-tocopherol (13,14,15) and may also act as signal transduction molecule (16). Hence it would be interesting to see the effect of γ-tocotrienol on DNA damage in lymphocytes of normal individuals and DS patients. DNA damage is evaluated using single-cell gel electrophoresis (SCGE) or comet assay. This assay has been reported to be a more sensitive assay to assess DNA damage than chromosomal aberration assessment at metaphase (17). In order to determine if γ-tocotrienol influences DNA repair, basal and caffeine -treated cells will be evaluated. Caffeine inhibits the G2 DNA repair phase and thus provide endogenous DNA damage level.

Materials and methods
This study was approved by the Universiti Kebangsaan Malaysia Ethics Committee. γ-Tocotrienol extracted from palm oil was a gift from Malaysia Palm Oil Board. Blood samples were collected from 11 healthy normal and 8 Down syndrome patients aged 1-7 years old.

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Lymphocyte culture and drug treatment

200 µl whole blood samples was added to 12 well plate containing 1 ml complete culture media (RPMI folate free, GIBCO, USA). The experiment was conducted according to that described by Pincheira et al (11) except that 20 µM γ-tocotrienol (GTT) was used in place of 100 µM α-tocopherol. The concentration of GTT was determined earlier in preliminary experiments. Thus four groups of cultures were set up from the sample of each donor. Two of them were treated with 20 µM GTT and incubated for 72 h at 37°C. In the last 2 h of incubation, caffeine treatments at a final concentration of 5 mM were given to two culture bottles (one untreated and one treated with GTT). Cells were harvested and DNA damage analysed using the alkaline single-cell gel electrophoresis.

Single-cell gel electrophoresis (SCGE; comet assay)

The alkaline version of comet assay was performed according to the method described by Collins et al. (18). Cells were embedded in 75 µl low-melting point agarose (0.6%, Boehringer Mannheim, Germany) (DNAse free, RNase free) layer on agarose-coated frosted slides. The cells were lysed in a buffer containing 2.5 M NaCl, 100 mM EDTA, 10mM Tris, 1% N-lauroylsarcosine, 1% Triton and 2% dimethylsulphoxide, at pH 10, for 1 hour at 4°C. Thereafter, the cells were exposed to a strong alkali solution (300 mM NaOH, 1 mM EDTA, pH 13) for 25 min in an electrophoresis chamber. Electrophoresis was performed for 20 min at 25 V and 300 mA. The slides were then neutralized (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 µg/ml). Comets were analysed by fluorescence microscopy (Carl Zeiss, Germany) with visual inspection of tail length of nuclei. The cell nuclei were classified into four categories: (i) undamaged (nuclei without comet tail); (ii) low damage (nuclei with comet tails up to twice); (iii) damaged (nuclei with comet tail longer than twice and up to threefold), and (iv) highly damaged (nuclei with comet tails longer than threefold the diameter of the nucleus), respectively. At least 1 X 80 cells per slide were evaluated.

Statistical analysis

Data are expressed as mean value ± standard deviation (SD). Statistical significance was calculated using the Student’s chi square test.

Results

The single-cell gel electrophoresis (SCGE), also known as comet assay, was first developed by Ostling and Johanson in 1984 (19). The method is based on the electrophoresis of cells embedded and lysed in agarose on a microscope slide. The underlying mechanism is that the DNA is organized in large supercoiled structures which, when relaxed by strand breaks in the DNA, could be stretched out by electrophoresis. This method provides a rapid, visual method for quantitatively assessing DNA breakage in single cells. It also has the advantage of measuring recent DNA damage in contrast to micronucleus analysis which measures damage accumulated over a long period of time (17). Thus it is a method of choice to determine the protective effects of test substances in culture.

Figures 1 and 2 represent percent number of cells with damaged DNA in lymphocyte cultures from normal and DS subjects respectively. The data shows that the number of cells with damaged DNA was significantly higher in lymphocyte culture from DS (p< 0.001) than normal subjects. The number of cells with more severe DNA damage were higher in DS (Fig 2) compared to normal lymphocytes (Fig 1).

The figures also show that there was a significant decrease (p<0.001) in the number, as well as the severity, of DNA damage in lymphocytes from DS and normal subjects (p<0.05) pretreated with GTT when compared to untreated cells.

There were significant increase in the number and the severity of DNA damage when cells were exposed to caffeine. However, pretreatment with GTT before the exposure to caffeine, significantly reduced the number (p<0.05) and severity of DNA damage.

![Figure 1: Means (±SD) number of cells with damaged DNA (%) in lymphocyte cultures from normal individuals treated with either gamma-tocotrienol (GTT) and / or caffeine (n=11).](image-url)
Vit E reduces DNA damage in Downs syndrome

Discussion

Previous reports on DNA damage in DS cells have been confusing. While some researchers reported increased DNA damage in DS cells (11,17), others did not observe any difference between DS and normal cells (20,21). The present data confirmed that DNA damage is higher in cells taken from DS patients than from normal individuals. Increased in DNA damage in DS has been associated with high H2 O2 production and hence hydroxyl radicals, resulting from the increase dosage of Cu/Zn SOD (22). Hydroxyl radicals not only oxidise bases but may also generate strand interruptions (23). Furthermore peroxidation of polyunsaturated lipids generates substances such as lipid hydroperoxides and radicals, eg. alkoxy and peroxyl, which possess DNA damaging potential (24). Lipid peroxidation also produces a range of carbonyl-containing compounds such as malondialdehyde that give rise to exocyclic adducts (23,24).

Increased DNA damage might also be due to decreased DNA repair capability in DS cells (7,10,25). ROS may interfere with DNA repair mechanism or it may exhaust the repair capability of cells (17). Alternatively, this might be due to over expression of several genes found on the DS critical region of chromosome 21. Over expression of cystathione beta synthase (CBS) and GART for example, may disrupt DNA repair and synthesis (26,27).

Treatment of cells with GTT resulted in a reduction in DNA damage under basal as well as when DNA repair is altered by caffeine treatment in G2. Pincheira et al. (11) treated lymphocytes from DS and controls with α-tocopherol, another subfamily of vitamin E, and measured chromosomal aberrations under basal and caffeine induced inhibition of DNA repair and reported similar findings. However the mechanism involved in the detection of DNA damage differs from the present method which employs SCGE that detects reparable DNA breakage and alkali-labile sites.

γ-Tocotrienol, an antioxidant, may act by quenching free radicals and improving the antioxidant status of the cells. This in turn, may decrease the oxidative DNA damage as reflected by the decrease in DNA damage index and severity when cells were pretreated with GTT before caffeine treatment. The decrease in endogenous DNA damage improves repair efficiency of these cells as is shown by the decrease in basal DNA damage with GTT treatment. However vitamin C, another antioxidant vitamin was reported to offer no significant protection against DNA damage induced by hydrogen peroxide and γ-irradiation (28). Probably the protective effect of γ-tocotrienol against DNA damage may not only be due to their potent antioxidant properties but may also involve their roles in signal transduction. Clearly more work is needed to elucidate the mechanism of action of γ-tocotrienol in this respect.

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References