α-Tocopherol supplementation reduces biomarkers of oxidative stress in children with Down syndrome: a randomized controlled trial

S Mustafa Nachvak1,2, T Reza Neyestani3, S Ali Mahboob4, S Sabour4, S Ali Keshawarz5 and JR Speakman2,6

BACKGROUND: Down syndrome (DS) is the most common human chromosomal abnormality. It is characterized by mental retardation and several metabolic disturbances, including elevated oxidative stress, which may be causally linked. Treatment with dietary antioxidants has been suggested as a potential method to alleviate the oxidative damage and retardation of DS patients, but prior supplementation work has been equivocal.

AIM: To evaluate the effects of supplementation with antioxidants α-tocopherol and α-lipoic acid (ALA) on oxidative stress biomarkers in DS children.

METHODS: Ninety-three DS children aged 7–15 years from both sexes were randomly allocated to three groups: α-tocopherol (400 IU/day), ALA (100 mg/day) and placebo. The intervention period was 4 months. A healthy control group consisted 26 non-DS siblings. Serum thiobarbituric acid reactive substances (TBARS) and urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) were used as biomarkers of oxidative stress.

RESULTS: DS children had greater levels of baseline oxidative stress than their siblings. Moreover, males had greater levels of 8OHdG than females (P < 0.001) but there was no significant association between age and biomarkers of oxidative stress. Serum levels of TBARS did not change significantly over time, or relative to placebo. Although urinary 8OHdG concentrations decreased significantly in both α-tocopherol and ALA, groups compared with the baseline levels (P < 0.001), mean final levels of urinary 8OHdG concentrations differed significantly only between α-tocopherol and placebo groups (P < 0.01).

CONCLUSIONS: α-Tocopherol supplementation of the diets of DS children may attenuate oxidative stress at the DNA level.

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INTRODUCTION

Down syndrome (DS) or Trisomy 21 is the most common chromosome abnormality in humans. Although many of the phenotypic consequences of DS show large individual variation, three common patterns are observed in almost all individuals. These are neuromotor dysfunction coupled with and cognitive impairment and retarded language development. Currently, there are no treatments available for these impairments. Consequently DS is the most common cause of mental retardation and is responsible for more than a third of learning disorders in human societies. The incidence of DS is 1 in 700 live births, which is the same among all racial groups and different socio-economic levels.

Mental retardation is the main clinical outcome in DS. The pathology of mental retardation in this syndrome is not completely understood, but it has been hypothesized that mental retardation in DS is the result of death and damage to neuronal cells due to oxidative stress. This increased stress may come about because one of the key enzymes involved in detoxification of radical oxygen species (Cu-Zn superoxide dismutase: SOD I) is located on chromosome 21. There is consequently a gene dosage effect in DS leading to 50% greater levels of SOD I and elevated production of hydrogen peroxide (H2O2), potentially leading to greater levels of the damaging hydroxyl radical via the Fenton reaction. The high production of H2O2 and resultant damage potential may be exacerbated by the very low consumption of fruit and vegetables in DS children which otherwise provide protective non-enzymatic antioxidants. In theory, treating DS children with dietary antioxidants may offset the elevated H2O2 production, ameliorate damage and thereby reduce the extent of cognitive impairment. The lipid-soluble α-tocopherol may be particularly useful in this respect because it blocks proliferation of membrane lipid peroxidation – a primary result of damage by the hydroxyl radical. A previous randomized controlled trial (RCT) of α-tocopherol treatment however revealed no impact of supplementation on either oxidative damage or cognitive performance in DS children. One problem with this previous trial, however, was that the experimental doses used were barely above the recommended daily allowance for α-tocopherol. However, a more recent trial in adults with DS and dementia using a much higher dose of antioxidants (900 IU α-tocopherol, 200 mg ascorbic acid and 600 mg α-lipoic acid) did not improve cognitive function or halt its decline. The study concluded that there were no adverse effects of this dosage level but it was an ineffective treatment. As by adulthood the neuronal damage in DS may be irreversible, but earlier intervention may prevent the damage happening.
there is still a need to verify in a RCT whether these high-dose treatments with antioxidants are effective or not in children with DS.

The aim of this study was to investigate the effect of dietary supplementation of α-tocopherol and α-lipoic acid (ALA) on oxidative stress in children with DS.

**MATERIALS AND METHODS**

Subjects and study design

This study was a RCT performed to evaluate the effects of dietary RRR-α-tocopherol and ALA supplements for reducing oxidative stress in children with DS. The study flow chart is included as Figure 1 and the trial protocol is included in the Supplementary materials S1. At the outset, we aimed to have a sample of 50 children per group. This sample size was based on a power analysis; given the known interindividual coefficient of variation of the damage assay of approximately 15%, the sample size would give a power of 80% to detect an effect size of 6% using a paired test comparing before and after treatment measurements at an alpha of 0.05. The final study group consisted of 93 children with DS aged 7–15 years. All children were selected from 10 Special Education Centres for Mentally Handicapped Children in different districts of Tehran, Iran. After selection of the centers, parents were invited to attend an information session. We described the procedures and details of the trial and asked them to sign an informed consent form if they desired the participation of their children in the trial. One hundred and forty-six parents signed informed consent and the children were randomized into three equal groups using a random number generator on a laptop computer (n = 49, 49 and 48). Allocation was made without the parents present so they were unaware of the treatment allocations. The enrollment to the study and allocation to groups was performed by SMN. Presence of parents with their children at the time of blood sampling was one of the ethical requirements of the project. We could not take blood from 44 children because their parents did not attend at the scheduled times for blood sampling. Also, we failed to collect blood sample from 13 children at the beginning of the study and four children at the end of the intervention because they individually refused consent to provide the blood sample. We also recruited 26 non-DS siblings in the same age range as a healthy control group. The children with DS were evaluated by a pediatrician before enrollment in the study and were confirmed to have no associated anomalies or disease. In all the children, DS diagnosis was confirmed by cytogenetic analysis, which identified subjects with regular trisomy 21. Inclusion criteria for the study included that the children lived with their parents and were not on any medication or vitamin supplementation therapy for 6 months before the start of intervention. Children in the three groups were each given capsules to take daily. The children and their parents were blind to the intervention. In group 1, the capsule contained 400 IU RRR-α-tocopherol (DSM Ltd). In group 2, the capsule contained 100-mg ALA and in group 3 the capsule contained only corn oil as a placebo. Corn oil contains only trace levels of vitamin E (primarily α-tocopherol) with a content of approximately 1.9 mg per 15 g.9 hence about 0.06 mg per capsule compared with 266 mg in the α-tocopherol treatment capsule (4400x greater). Capsules were administered daily by the parents for a period of 4 months. The study was approved by the Ethics Committee of Tehran University of Medicine Sciences in accordance with Helsinki Declaration and guideline of Iranian Ministry of Health and Medical Education (IRB 2507).

Quantification of lipid damage by thiobarbituric acid reactive substances (TBARS)

Blood and urine samples were taken at baseline before supplementation and after 4 months of supplementation. We measured thiobarbituric acid reactive substances (TBARS) which consist mostly of malondialdehyde (MDA) in serum and 8-hydroxy-2'-deoxyguanosine (8OHdG) in urine samples as markers of oxidative stress. To determine TBARS concentrations, 5 ml blood was collected from children following an overnight fast. Serum was drawn after at least 30 min of clotting by centrifugation at 2500 g for 15 min. The determination of TBARS was performed by method of Satho.10 In this method, the reaction with Thiobarbituric acid (TBA) creates a complex, which is determined spectrophotometrically. Briefly, serum samples were mixed with trichloroacetic acid (20%) and the precipitate was dissolved in sulfuric acid (0.05 M). TBA (0.02% in sodium phosphate buffer) was added and the mixture was boiled for 5 minutes. After cooling, thiobarbituric acid-reactive substances (TBARS) were measured spectrophotometrically at 532 nm.

**Figure 1.** Trial flow chart.
sulfate 2 μl) was added and heated for 30 min in boiling water bath. TBA reactive substances (TBARS) adducts were extracted by n-butanol and absorbance was measured at 532 nm by UV-160-A Shimadzu double beam spectrophotometer (Kyoto, Japan).

Quantification of DNA damage by urinary 8OHdG

Ten ml spot morning urine samples were collected in polypropylene specimen tubes, as it proved difficult to collect 24-h urine samples in children with DS. Thompson et al.11 indicated that 24-h average urinary levels were not statistically different from first voids, so we decided to use the first morning voids rather than the 24-h collections. 8OHdG in urine samples was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (8OHdG Quantitation, Cell Biolabs, Inc. San Diego, CA, USA).12 The ELISA assay was performed according to the manufacturer’s instructions. Briefly, urine samples were centrifuged at 3000 g for 10 min; then, the supernatant was diluted with phosphate-buffered saline. A 50 μl aliquot of the primary antibody and 50 μl of the digested DNA and diluted urine samples were added to a micro plate that had been precoated with 8OHdG; then, they were incubated at room temperature for 1 h on an orbital shaker after which the plate was washed three times thoroughly with 250 μl washing solution. Each of the wells on the plate was then incubated at room temperature for 1 h with 100 μl horseradish peroxidase-conjugated secondary antibody and subsequently washed with 250 μl washing solution. In the next step, 100 μl enzyme-substrate solution was added and the plates were incubated at room temperature for 15 min on an orbital shaker. The enzyme reaction was stopped by adding 100 μl of stop solution (1 μl phosphoric acid) for about 3 min, after which the absorbance at 450 nm could be read using a Benchmark Microplate Reader (Bio-Rad Inc, Hercules, CA, USA). The above procedures were performed under dark conditions. Concentration of 8OHdG was calculated from a standard curve. All data were analyzed blind of the procedures were performed under dark conditions. Concentration of 8OHdG was calculated from a standard curve. All data were analyzed blind of the procedure.

RESULTS

Characteristics of the children with DS are shown in Table 1. No adverse effects were reported during the trial. Biomarkers of oxidative stress in children with DS were significantly higher than their non-DS siblings (Table 2; Figure 2). Moreover, in the DS group, males had higher 8OHdG levels than females (P < 0.001; Table 3). No significant relationship between age of the children and oxidative stress biomarkers was observed (regression, P > 0.05).

At the beginning of the study before the intervention started, there was no significant difference between means of serum TBARS and urinary 8OHdG concentrations among the three DS groups (ANOVA, P > 0.05). After the intervention, serum levels of TBARS were not significantly altered relative to baseline in any group. Although urinary 8OHdG concentrations showed a statistically significant decrease in both α-tocopherol and ALA groups compared with the initial levels (paired t-tests, P < 0.001), the final mean levels of urinary 8OHdG concentrations differed significantly only between the α-tocopherol and the placebo group (ANOVA followed by Tukey test, P < 0.05; Table 4; Figure 3). These data indicate that dietary supplementation using α-tocopherol at a dose of 400 IU/day over a period of 4 months might significantly reduce oxidative damage to DNA in DS children.

DISCUSSION

Our findings comparing DS children with their healthy siblings are in line with previous studies,13–16 which have shown greater levels of oxidative stress in DS children. One hypothesis for the repeated observations of greater oxidative stress in DS children is due to a gene dosage effect of Cu-Zn SOD I, which is an antioxidant enzyme located on chromosome 21 that converts the superoxide radical (O2-) to cytotoxic hydrogen peroxide (H2O2); SOD1 levels are increased by 50% in patients with DS17 leading to elevated levels of H2O2 and the highly toxic OH· radical possibly via the fenton reaction.19 In our study, urinary 8OHdG concentrations in boys with DS were significantly higher than in girls with DS. These data are consistent with a study that measured antioxidant gene expression levels in male and females with DS, which found the expression levels of antioxidant genes in females with DS was higher than in males with DS, suggesting that males with DS are more vulnerable to oxidative damage.20 The clinical significance of this effect, however, is uncertain because there is no suggestion that symptom severity, prognosis or occurrence of neurodegenerative complications are increased in DS boys compared with girls. In the present study, TBARS levels were not significantly different among the groups compared with the initial levels (paired t-tests, P > 0.05) which found that symptom severity, prognosis or occurrence of neurodegenerative complications are increased in DS boys compared with girls.

Table 1. Characteristics of the children with Down Syndrome

<table>
<thead>
<tr>
<th>Sex</th>
<th>N</th>
<th>%</th>
<th>Age (years)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boys</td>
<td>56</td>
<td>60.2</td>
<td>11.2 ± 2.7</td>
<td>0.343</td>
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<tr>
<td>Girls</td>
<td>37</td>
<td>39.8</td>
<td>11.7 ± 2.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Mean urinary 8OHdG (ng/ml) ± s.d. between boys and girls with Down Syndrome

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Sex</th>
<th>N</th>
<th>Mean</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>Boys</td>
<td>56</td>
<td>3.18 ± 0.45</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>Girls</td>
<td>37</td>
<td>2.88 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>Boys</td>
<td>56</td>
<td>2.84 ± 0.40</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>Girls</td>
<td>37</td>
<td>2.52 ± 0.35</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: 8OHdG, 8-hydroxy-2'-deoxyguanosine. *Significant at P < 0.05.
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Table 3. Comparisons of mean MDA and 8OHdG concentrations ± s.d. and age between children with DS and their siblings

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Mean MDA (nmol/ml)</th>
<th>P-value</th>
<th>N</th>
<th>Mean 8OHdG (ng/ml)</th>
<th>P-value</th>
<th>N</th>
<th>Mean age (years)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS</td>
<td>83</td>
<td>2.90 ± 0.78</td>
<td>0.001*</td>
<td>93</td>
<td>3.06 ± 0.46</td>
<td>0.001*</td>
<td>93</td>
<td>11.4 ± 2.5</td>
<td>0.496</td>
</tr>
<tr>
<td>Siblings</td>
<td>26</td>
<td>2.43 ± 0.55</td>
<td></td>
<td>26</td>
<td>2.26 ± 0.27</td>
<td></td>
<td>26</td>
<td>11.0 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: DS, Down Syndrome; MDA, malondialdehyde; 8OHdG, 8-hydroxy-2′-deoxyguanosine. *Significant at P < 0.05.

Table 4. Mean urinary 8OHdG (ng/ml) ± s.d. in the treatment groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Intervention</th>
<th>N</th>
<th>Mean 8OHdG (ng/ml)</th>
<th>P-value</th>
<th>Change of mean (ng/ml)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha tocopherol</td>
<td>Before</td>
<td>32</td>
<td>3.15 ± 0.51</td>
<td>0.001*</td>
<td>−0.5 ± 0.6</td>
<td>0.04a</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>32</td>
<td>2.66 ± 0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALA</td>
<td>Before</td>
<td>32</td>
<td>3.04 ± 0.45</td>
<td>0.001*</td>
<td>−0.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>32</td>
<td>2.66 ± 0.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>Before</td>
<td>29</td>
<td>2.99 ± 0.43</td>
<td>0.08</td>
<td>−0.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>29</td>
<td>2.83 ± 0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALA, α-lipoic acid; 8OHdG, 8-hydroxy-2′-deoxyguanosine. *significant at P < 0.05.

Figure 3. (a) Mean urinary 8OHdG (ng/ml) and (b) Mean serum MDA (nmol/ml) in the three study groups before and after intervention. VitE is vitamin E treated, ALA is α-lipoic acid treated and placebo received vehicle only. * indicates a significant difference between pre and post treatment groups at P < 0.05.

wavelength used to assay MDA levels.22 Hence, the measure lacks specificity. However, some of these aldehydes are also products of lipid peroxidation; so while not measuring specifically MDA the assay may in theory still provide a valid test of lipid peroxidation. Nevertheless, there are two additional issues with this assay. It has been suggested that the primary source of MDA detected by this method reflects mostly MDA generated by decomposition of lipid peroxides during the stage of the test where the compounds are heated in acid. This leads to a high background level making detection of biological effects more difficult. Moreover, additional compounds such as some sugars, amino acids and bilirubin may also react with TBA.23

In contrast to the results for TBARS, we found both ALA and α-tocopherol interventions significantly reduced urinary levels of 8OHdG relative to baseline, although the final levels were only different to the placebo-treated group for the α-tocopherol-supplemented children. These data indicate oxidative stress to DNA might be reduced in DS by supplementation of α-tocopherol at a level of 400 IU per day. Immunochemochemical detection of injury to DNA by ELISA is a method in widespread use,26 yet it is also not without issues, mostly related to specificity of the antibodies employed. ELISA-based assays of 8OHdG of DNA extracted from tissues are reported to correspond closely to levels measured by high-performance liquid chromatography (HPLC). Two independent studies reported correlation coefficients of r = 0.96 for analyses of thymus- and placenta-derived DNA.27,28 The absolute levels detected by ELISA were however sixfold higher by ELISA than by HPLC. It has been suggested that this is because antibodies for 8OHdG also cross-react with other damaged nucleobases such as 8-oxo-7,8-dihydroguanine that are structurally similar.27 The use of ELISA to detect 8OHdG levels in biological fluids, particularly serum, has been questioned.25 Nevertheless, measurements of 8OHdG in urine using ELISA are reasonably well correlated with measurements based on HPLC separation followed by tandem mass spectrometry (t = 0.84),27 despite the fact levels by ELISA always greatly exceed those by HPLC, possibly because of some interference by urea levels.26 Consequently, sensitivity of the ELISA method, and hence the ability to separate differences between treatment groups is lower with ELISA than HPLC.27 Interlaboratory comparisons using ELISA assays of urine suggest good repeatability across labs.27 The trend for reduced damage under ALA treatment detected here therefore warrants further investigation in the future using more sensitive methods.
Our results contrast the results of a previous RCT in children to evaluate the effects of supplementation with antioxidants, folic acid, or both to reduce oxidative damage and improve the psychomotor and language development of children with DS.7 This previous RCT provided no evidence to support the use of antioxidant or folic acid supplements in children with DS. However, a major drawback of this previous study was that the supplementation was at a level similar to the recommended daily allowance. The main difference between these studies is consequently the dosage of antioxidants used. A subsequent study8 used a much higher dose of antioxidants in adults (900 IU α-tocopherol, 200 mg ascorbic acid and 600 mg α-lipoic acid) and concluded that this treatment did not halt or reverse cognitive decline. Our data contrasts these latter data and suggest that doses around 400 IU/day may provide significant reductions in oxidative stress in DS children. Although using very high doses of α-tocopherol has raised concerns,9 the level at which dietary doses appear to generate negative effects on health are considerably higher than the 400 IU/day used here. Indeed, other studies have suggested that doses of over 1600 IU/day over periods exceeding 16 weeks may be necessary to generate reductions in oxidative damage to lipids as measured by f2-isoprostanes.10 Hence, another reason why we failed to observe reductions in oxidative damage to lipids as measured by α-tocopherol was a generous unrestricted gift from DSM Ltd (Netherlands). The trial was given ethical approval by the Ethics committee of the Tehran University of Medical Sciences and registered with the Iranian Clinical Trials Registry (registration number ICT201302215111N1).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES

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