European Journal of Clinical Nutrition (2014), 1–5

www.nature.com/ejcn

ORIGINAL ARTICLE

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

S Mustafa Nachvak[1,2], T Reza Neyestani[3], S Ali Mahboob[4], S Sabour[4], S Ali Keshawarz[5] and JR Speakman[2,6]

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.

European Journal of Clinical Nutrition advance online publication, 18 June 2014; doi:10.1038/ejcn.2014.97

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.[1] The incidence of DS is 1 in 700 live births, which is the same among all racial groups and different socio-economic levels.[2]

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.[3–5] 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 (H_2O_2), potentially leading to greater levels of the damaging hydroxyl radical via the Fenton reaction. The high production of H_2O_2 and resultant damage potential may be exacerbated by the very low consumption of fruit and vegetables in DS children[6] which otherwise provide protective non-enzymatic antioxidants. In theory, treating DS children with dietary antioxidants may offset the elevated H_2O_2 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[7] 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.[8] 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,

[1]Faculty of Public Health, Kermanshah University of Medical Sciences, Kermanshah, Iran; [2]Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK; [3]National Nutrition and food technology Research Institute (NNFTRI), Iran; [4]Nutrition research Center, Tabriz University of medical sciences, Tabriz, Iran; [5]Tehran University of Medical Sciences, Tehran, Iran and [6]State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. Correspondence: Dr JR Speakman, Institute of Biological and Environmental Science, University of Aberdeen, Tillydrone Ave, Aberdeen, ScotlandAB24 2TZ, UK. E-mail: J.Speakman@abdn.ac.uk
Received 5 December 2013; revised 10 April 2014; accepted 13 April 2014

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 Supplementory 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 co-efficient of variation of the damage assays 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

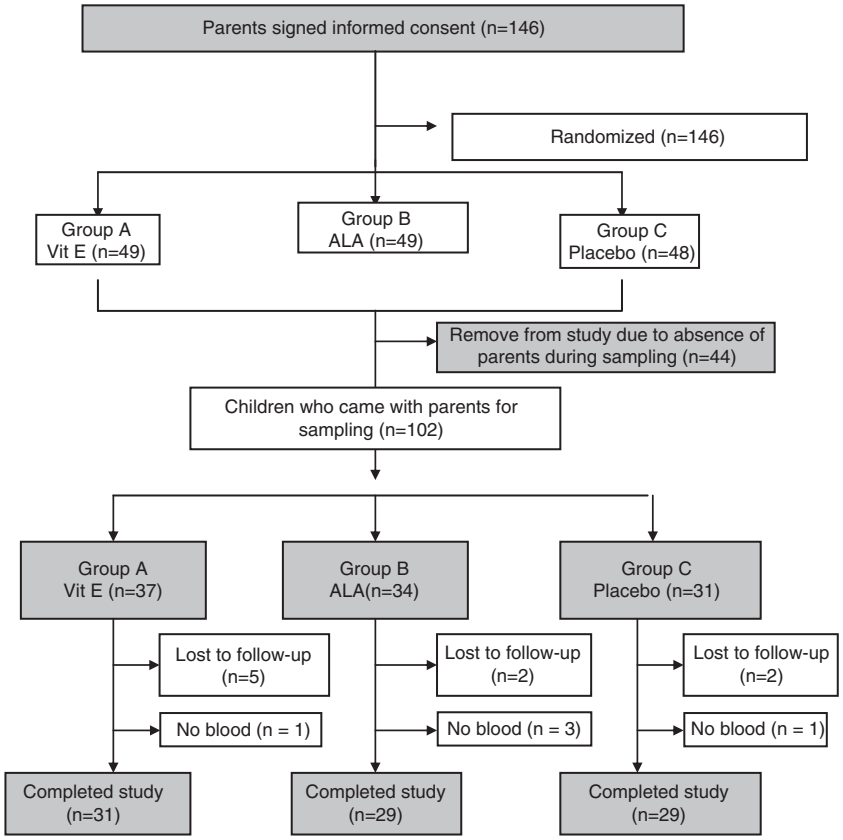

Figure 1. Trial flow chart.

sulfate 2 M) 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 second 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 M 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 intervention groupings. We did not normalize the data for creatinine levels as it has been suggested that renal malfunctions in DS[11] may reduce excreted creatinine levels and thus spuriously elevate levels of other markers relative to creatinine.

Statistics

Although we aimed to recruit 50 children per group we ended up with around 30 per group. Dropouts were not differentially biased according to treatment (Figure 1) and hence we performed a per protocol analysis. With a final sample of around 30 per group, we had a power of 80% to detect an effect size of 8% using a paired test at $\alpha = 0.05$. Results are expressed as mean ± s.d. Data were checked for normality before analysis. For comparison of the groups, Paired sample *t*-test, Student's *t*-test and analysis of variance (ANOVA) were performed. When ANOVA was significant, we used *post hoc* Tukey tests to compare group means. Pearson's correlation was used to establish linear correlations between variables. A *P*-value less than 0.05 was considered significant. Analyses were carried out using the Statistical Package for Social Sciences (SPSS 15.00 for windows, IBM Ltd, Armonk, NY, USA).

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.

Table 1. Characteristics of the children with Down Syndrome

Sex	N	*%*	*Age (years)*	P-*value*
Boys	56	60.2	11.2 ± 2.7	0.343
Girls	37	39.8	11.7 ± 2.3	

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

Intervention	*Sex*	N	*Mean*	P-*value*
Before	Boys	56	3.18 ± 0.45	0.002[a]
	Girls	37	2.88 ± 0.43	
After	Boys	56	2.84 ± 0.40	0.001[a]
	Girls	37	2.52 ± 0.35	

Abbreviation: 8OHdG, 8-hydroxy-2′-deoxyguanosine. [a]Significant at $P < 0.05$.

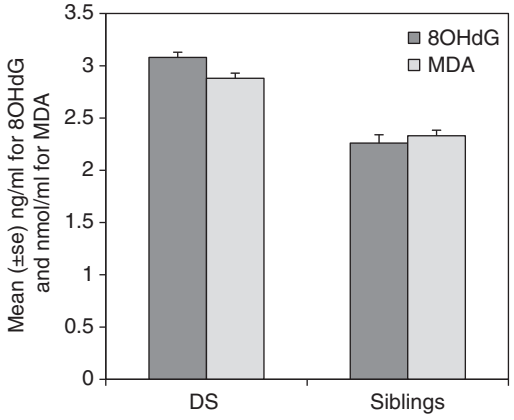

Figure 2. Oxidative stress biomarkers in Down syndrome children and their siblings at baseline before antioxidant treatment. Error bars are standard errors.

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 (O_2^-) to cytotoxic hydrogen peroxide (H_2O_2). SOD1 levels are increased by 50% in patients with DS[17] leading to elevated levels of H_2O_2[18] 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

Table 3. Comparisons of mean MDA and 8OHdG concentrations ± s.d. and age between children with DS and their siblings

Groups	N	*Mean MDA*	P-*value*	N	*Mean 8OHdG*	P-*value*	N	*Mean age*	P-*value*
DS	83	2.90 ± .78	0.001[a]	93	3.06 ± .46	0.001[a]	93	11.4 ± 2.5	0.496
Siblings	26	2.43 ± 55		26	2.26 ± .27		26	11.0 ± 2.2	

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

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

Groups	*Intervention*	N	*Mean*	P-*value*	*Change of mean*	P-*value*
Alpha tocopherol	Before	32	3.15 ± 0.51	0.001[a]	− 0.5 ± 0.6	0.04[a]
	After	32	2.66 ± 0.39			
ALA	Before	32	3.04 ± 0.45	0.001[a]	− 0.3 ± 0.5	
	After	32	2.66 ± 0.43			
Placebo	Before	29	2.99 ± 0.43	0.08	− 0.1 ± 0.4	
	After	29	2.83 ± 0.40			

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

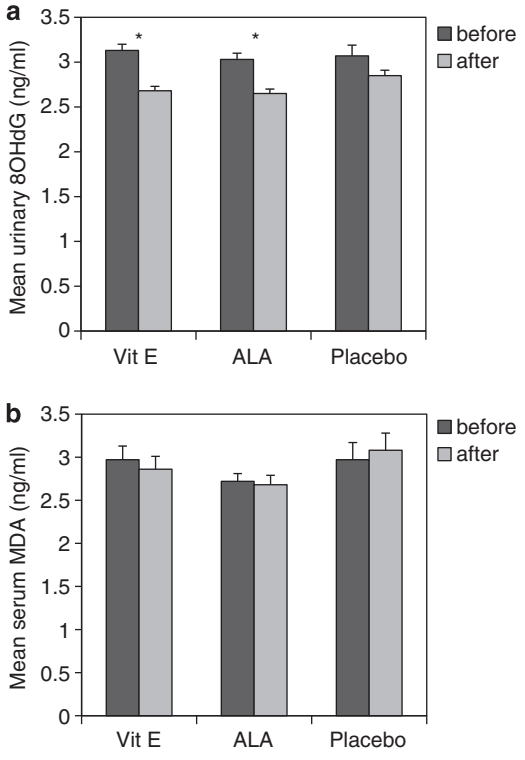

Figure 3. (**a**) Mean urinary 8OHdG (ng/ml) and (**b**) Mean serum MDA (nmol/ml) in the three study groups before and after intervention. vitE is vitamin E treated, ALA is α-lipoic acid treated and placebo received vehicle only. * indicates a significant difference between pre and post treatment groups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

altered by either of the antioxidant interventions. This could be because the intervention did not protect against lipid damage or alternatively because the Thiobarbituric acid (TBARS) assay, although in widespread use, has several limitations.[21,22] In particular, the assay may work when applied to defined systems such as liposomes but is less reliable when used to measure body fluids, as was performed here.[23] This is in part because the primary marker of lipid damage MDA is not the only aldehyde that can react with TBA to form chromogens that absorb at the 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. Immunochemical detection of damage to DNA by ELISA is a method in widespread use,[24] 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.[25,26] 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 ($r = 0.84$),[27] despite the fact levels by ELISA always greatly exceed those by HPLC, possibly because of some interference by urea levels.[28] 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.[29] 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, folinic 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 folinic 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 study[8] 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 contrast 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,[30] 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.[31] Hence, another reason why we failed to observe an effect on lipid damage may have been because the dose of α-tocopherol we used may have been too low. Lott *et al.*[8] concluded that while the dose of 900 IU α-tocopherol was ineffective, it did not generate any adverse effects and was therefore safe. The main limitations of this trial were the final sample size, which eroded some of the intended power, and the quality of the lipid peroxidation assay we used. Nevertheless, our data support instigation of a larger and longer trial at the current dosage level of 400 IU, or higher doses, to explore a wider range of oxidative damage markers in addition to potential impacts on cognitive development in DS children.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the families and children involved in our study. We also thank Dr Ahmad Reza Dorosti, Dr Reza Mahdavi, Dr Majid Hajifaraji, Dr Mehdi Hedaiati, Dr Mansoor Rezaei and Mrs Nasrin Shariatzadeh for their valuable contributions. This study was supported in part by National Nutrition and Food Technology Research Institute (NNFTRI). The 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 IRCT2013102215111N1).

REFERENCES

1 Iles RK. Cell and molecular biology, and genetic disorders. In Kumar, P, Clark, M. (eds) *Clinical Medicine*. 5th edn. W. B. Saunders: Edinburgh, UK, 2004, pp 172–173.
2 Mégarbané A, Ravel A, Mircher C, Sturtz F, Grattau Y, Rethoré MO *et al.* The 50th anniversary of the discovery of trisomy 21: the past, present, and future of research and treatment of Down syndrome. *Genet Med* 2009; **11**: 611–616.
3 de Haan JB, Susil B, Pritchard M. An altered antioxidant balance occurs in Down syndrome fetal organs. *J Neural Transm Suppl* 2003; **67**: 67–83.
4 Nagy Z. Mechanisms of neuronal death in Down's syndrome. *J Neural Transm suppl* 1999; **57**: 223–245.
5 Muchova J, Sustrova M. Influence of age on activities of antioxidant enzymes and lipid peroxidation products in erythrocytes and neutrophils of Down syndrome patients. *Free Radical Biol Med* 2001; **31**: 499–508.
6 Nachvak SM, Mahboob SA, Speakman JR. Low consumption of fruit and vegetables, and markers of oxidative stress in children with Down syndrome. *Downs Syndr Res Pract* 2010; **13**.
7 Ellis JM, Tan HK, Gilbert RE, Muller DP, Henley W, Moy R *et al.* Supplementation with antioxidants and folinic acid for children with Down's syndrome: randomised controlled trial. *BMJ* 2008; **336**: 594–597.
8 Lott IT, Doran E, Nguyen VQ, Tournay A, Head E, Gillen DL. Down syndrome and dementia: a randomized, controlled trial of antioxidant supplementation. *Am J Med Genet A* 2011; **155A**: 1939–1948.
9 Gallagher, ML. The nutrients and their metabolism.Mahan , LK, Escott Stump, S (eds). *Krause's Food and the Nutrition Care Process*. 13th edn Saunders: Pennsylvania, USA, 2012, pp 32–125.
10 Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by new colorimetric method. *Clinical Chimica Acta* 1978; **90**: 37–13.
11 Thompson HJ, Heimendinger J, Haegele A, Sedlacek SM, Gillette C, O'Neill C *et al.* Effect of increased vegetable and fruit consumption on markers of oxidative cellular damage. *Carcinogenesis* 1999; **20**: 2261–2266.
12 Guzmán R, Campos C, López-Fernández E, Casado A. Biomarkers of age effect on renal function in Down syndrome. *Biomarkers* 2011; **16**: 679–685.
13 Jovanovic SV, Clements D, MacLeod K. Biomarkers of oxidative stress are significantly elevated in Down syndrome. *Free Radic Biol Med* 1998; **25**: 1044–1048.
14 Zitnanová I, Korytár P, Aruoma OI, Sustrová M, Garaiová I, Muchová J *et al.* Uric acid and allantoin in Down syndrome: antioxidant and oxidative stress mechanism. *Clin Chim Acta* 2004; **341**: 139–146.
15 Carratelli M, Porcaro L, Ruscica M, De Simone E, Bertelli AA, Corsi MM. Reactive oxygen metabolites and pro-oxidant status in children with Down's syndrome. *Int J Clin Pharmacol Res.* 2001; **21**: 79–84.
16 Pinto M, Neves J, Palha M, Bicho M. Oxidative stress in Portuguese children with Down syndrome. *Downs Syndr Res Prac* 2002; **8**: 79–82.
17 Garces ME, Peres W, Salvador M. Oxidative stress and hematologic and biochemical parameters in individuals with Down syndrome. *Mayo Clin Proc* 2005; **80**: 1607–1611.
18 Midorikawa K, Kawanishi S. Superoxide dismutases enhance H2O2-induced DNA damage and alter its site specificity. *FEBS Lett.* 2001; **495**: 187–190.
19 Goldstein S, Meyerstein D, Czapski G. The Fenton reagents. *Free Radic Biol Med* 1993; **15**: 435–445.
20 Sinha S. Anti-oxidant gene expression imbalance, aging and Down syndrome. *Life Sci* 2005; **76**: 1407–1426.
21 Meagher EA, FitzGerald GA. Indices of lipid peroxidation *in vivo*: strengths and limitations. *Free Radic Biol Med* 2000; **28**: 1745–1750.
22 Kosugi H, Kato T, Kikugawa K. Formation of yellow, orange and red pigments in the reaction of alk-2-enals with 2-thiobarbituric acid . *Anal Biochem* 1987; **165**: 456–464.
23 Gutteridge HTW. Aspects to consider when detecting and measuring lipid peroxidation. *Free Radic Res Commun* 1986; **1**: 173–184.
24 Rossner P, Sram RJ. Immunochemical detection of oxidatively damaged DNA. *Free Radic Res* 2012; **46**: 492–522.
25 Breton J, Sichel F, Bianchini F, Prevost V. Measurement of 8-hydroxy-2′-deoxyguanosine by a commercially available ELISA test: comparison with HPLC/electrochemical detection in calf thymus DNA and determination in human serum. *Anal Lett* 2003; **36**: 123–134.
26 Yin B, Whyatt RM, Perera FP, Randall MC, Jedrychowski W, Cooper Y, Santella RM. Determination of 8-hydroxydeoxyguanosine by immunoaffinity chromatography-monoclonal antibody based ELISA. *Free Radic Biol Med* 1995; **18**: 1023–1032.
27 Hu CW, Wu MT, Chao MR, Pan CH, Wang CJ, Swenberg JA *et al.*. Comparison of analyses of urinary 9-hydroxy-2′-deoxyguanosine by isotope dilution liquid chromatography with electrosprat tandem mass spectrometry and by enzyme-linked immunosorbent assay. *Rapid Commun Mass Sp* 2004; **18**: 505–510.
28 Song MF, Li YS, Ootsuyama Y, Kasai H, Kawai K, Ohta M *et al.* Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to over-estimation of urinary 8-OH-dG. *Free Radic Biol Med* 2009; **47**: 41–46.
29 Evans MD, Olinski R, Loft S. Toward consensus in the analysis of urinary 8-oxo 7,8-dihydro-2′-deoxyguanosine as a non-invasive biomarker of oxidative stress. *FASEB J* 2010; **24**: 1249–1260.
30 Miller ER, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 2005; **142**: 37–46.
31 Roberts LJ, Oates JA, Linton MF, Fazio S, Meador BP, Gross MD *et al.* The relationship between dose of vitamin E and suppression of oxidative stress in humans. *Free Radic Biol Med* 2007; **43**: 1388–1393.

Supplementary Information accompanies this paper on European Journal of Clinical Nutrition website (http://www.nature.com/ejcn)