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ORIGINAL ARTICLE

Increased telomerase activity and vitamin D supplementation in overweight African Americans

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OBJECTIVE: We aimed to investigate whether vitamin D supplementation modulates peripheral blood mononuclear cell (PBMC) telomerase activity in overweight African Americans.
DESIGN: A double blind, randomized and placebo-controlled clinical trial (#NCT01141192) was recently conducted.
SUBJECTS AND METHODS: African-American adults were randomly assigned to either the placebo, or the vitamin D group (60 000 IU per month (equivalent to ~2000 IU per day) oral vitamin D3 supplementation). Fresh PBMCs were collected from 37 subjects (18 in the placebo group and 19 in the vitamin D group), both at baseline and 16 weeks. PBMC telomerase activity was measured by the telomeric repeat amplification protocol.
RESULTS: Serum 25 hydroxyvitamin D levels increased from 40.7 ± 15.7 at baseline to 48.1 ± 17.5 nmol l^{-1} at posttest ($P = 0.004$) in the placebo group, and from 35.4 ± 11.3 at baseline to 103.7 ± 31.5 nmol l^{-1} at posttests ($P < 0.0001$) in the vitamin D group. In the vitamin D group, PBMC telomerase activity increased by 19.2% from baseline (1.56 ± 0.29 absorbance reading unit (AU)) to posttest (1.86 ± 0.42 AU, $P < 0.0001$). The significance persisted after controlling for age, sex and body mass index ($P = 0.039$). PBMC telomerase activity in the placebo group did not change from baseline (1.43 ± 0.26 AU) to posttest (1.46 ± 0.27 AU, $P = 0.157$).
CONCLUSION: Vitamin D supplementation significantly increased PBMC telomerase activity in overweight African Americans. Our data suggest that vitamin D may improve telomere maintenance and prevent cell senescence and counteract obesity-induced acceleration of cellular aging.

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Keywords: vitamin D supplementation; telomerase activity; 25(OH)D; African Americans

INTRODUCTION

Telomeres, specialized DNA structures located at the chromosome ends, protect chromosome integrity and stability. Telomeres naturally shorten with every cell cycle, and cells with critically short telomeres undergo replicative senescence and apoptosis. Oxidative stress and inflammation dramatically decrease telomerase activity and accelerate telomere shortening. Obesity is characterized as a state of chronic inflammation and heightened oxidative stress. Several large studies have shown that shorter telomeres are associated with obesity in adulthood, suggesting that obesity accelerates cellular aging.[1–4] Telomerase is an essential enzyme that maintains telomere length and cellular replicate potential. Sustained telomerase activity stabilizes the telomere length and delays/prevents the replicative senescence of T cells.[5,6] Importantly, increasing telomerase activity not only affects the telomeres and proliferative potential but also preserves healthy cell function and long-term immune function.[7,8] Moreover, evidence from mouse models suggests that telomerase deficiency is associated with increased risk of cardiovascular disease,[9–12] impaired glucose metabolism and insulin secretion.[13]

Telomerase activity measured in the peripheral blood mononuclear cell (PBMC) samples constitutes a relatively new parameter, which has been measured only in a very few studies in humans to date. For example, resting PBMC telomerase activity is elevated in major depression and predicts antidepressant responses.[14] Low PBMC telomerase activity was associated with increased cardiovascular risk factors in 62 healthy women, suggesting that low leukocyte telomerase activity constitutes an early marker of cardiovascular risk.[15]

Recent studies highlight the beneficial effects of positive lifestyle changes on the cellular aging process, which is associated with telomere/telomerase decay. Farzaneh-Far *et al.*[16] recently showed that individuals in the lowest quartile of omega-3 fatty acids (docosahexaenoic acid and eicosapentaenoic acid) experienced the fastest telomere shortening, whereas those in the highest quartile experienced the slowest telomere shortening over 5 years. The pioneer comprehensive life style intervention study conducted by Ornish *et al.*[17] showed that PBMC telomerase activity had increased by 29% by the end of a 3-month lifestyle intervention in 24 low-risk prostate cancer patients.

Vitamin D is considered to have an important role in a broad range of bodily function beyond bone health, including immune function and cardiovascular health.[18–20] Vitamin D deficiency, commonly present in obese and African Americans,[21] is associated with various aging-related diseases, including hypertension, type 2 diabetes mellitus, cardiovascular diseases and all-cause mortality.[19,20,22–26] We recently demonstrated that vitamin D supplementation improved arterial stiffness[27] and endothelial function[28]

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in African Americans. A cross-sectional study suggests that higher serum 25 hydroxyvitamin D(25(OH)D) level is associated with longer leukocyte telomere length in 2160 Caucasian women.[29] Therefore, we aimed to explore the hypothesis that vitamin D supplementation increases PBMC telomerase activity in humans.

SUBJECTS AND METHODS

Subjects

A total of 70 African-American subjects (aged 19–50 years) reported to the Georgia Prevention Institute for onsite screening. Only apparently healthy, African-American men and women, who denied a history of cardiovascular, pulmonary or metabolic disease, were included in the study. Subjects were excluded if they (1) had evidence of diabetes (screening hemoglobin A1c $\geqslant$ 6.5 %) or GI/malabsorptive disorders, (2) were taking any medications known to affect calcium and/or vitamin D metabolism, (3) were taking any vasoactive medications, (4) were taking any vitamin, mineral, or herbal supplements, (5) were unable to swallow pills, and (6) were pregnant. Of the 70 subjects screened, 57 were found to be eligible and subsequently randomized into the study. All subjects provided written informed consent before study initiation. The protocol was approved by the Human Assurance Committee of the Georgia Health Sciences University.[28]

Experimental design

A double-blind, randomized, placebo-controlled clinical trial (#NCT01141192) was conducted. Following screening, all eligible subjects were randomly assigned to either the vitamin D group or the placebo group by the Georgia Health Sciences University's Clinical Research Pharmacy. Following the baseline assessments, all the subjects were given investigator-supervised dosing during their visits to Georgia Prevention Institute at every 4 weeks for a total of 16 weeks. The doses were dispensed by clinical research pharmacy of Georgia Health Sciences University. For each dosing visit, the experimental group received a 60 000 IU oral vitamin D_3 pill equivalent to 2000 IU per day (Bio-Tech Pharmacal Inc., Fayetteville, AR, USA) whereas the control group received an identical appearing placebo pill from the same manufacturer (microcrystalline cellulose, fumed silica). A dose of 2000 IU per day vitamin D3, previously recognized as the tolerable upper intake level, was considered to have no adverse health effects.[30]

Testing protocol

Testings at baseline and 16 weeks included anthropometric assessments of height, weight and body mass index (BMI). Resting blood pressure was evaluated in all subjects using established protocols.[31] Total cholesterol, high density lipoproteins, low-density lipoproteins (LDL) and triglycerides were obtained using the Cardiochek device (Polymer Technology Systems, Indianapolis, IN, USA). Glycocylated hemoglobin (hemoglobin A1c) was determined using the Bayer A1cNow+ point of care device (Bayer HealthCare LLC, Sunnyvale, CA, USA).

Biochemical analysis of 25(OH)D, parathyroid hormone (PTH) and calcium

Serum 25(OH)D, serum intact PTH, serum calcium (Ca^{2+}), and urine Ca^{2+} were measured at baseline and 16 weeks. Serum 25(OH)D concentrations were determined using an enzyme immunoassay (Immunodiagnostic Systems, Fountain Hills, AZ, USA) according to the manufacturer's instructions. Analytical reliability of the 25(OH)D assays was monitored through participation in DEQAS (Vitamin D External Quality Assessment Scheme) and was deemed acceptable. The intra- and inter-assay coefficient of variation (CV) for serum 25(OH)D were 5.9% and 6.6%, respectively. Bioactive intact PTH was determined in serum using ELISA (Immutopics, Inc., San Clemente, CA, USA) according to the manufacturer's instructions. The intra-assay CV for serum PTH was 5.7%. Both serum and urine Ca^{2+} were determined using the BioVision Colorimetric Calcium Assay Kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions. The intra-assay CVs for serum and urine calcium were 5.9% and 6.6%, respectively.

Detection of telomerase activity by the telomeric repeat amplification protocol

PBMCs were isolated from whole blood by Ficoll-Pague Premium (Sigma-Aldrich, St Louis, MO, USA) gradient centrifugation within 1 h after blood draw. Isolated PBMCs were stored in a cryopreservation media composed of RPMI-1640, 10% dimethyl sulfoxide and 10% fetal bovine serum at liquid nitrogen tank until further processing.

Telomerase activity was assayed by the Telo TAGGG Telomerase PCR ELISA kit (Roche Applied Science, Indianapolis, IN, USA). PBMCs were counted with a hemocytometer (BrightLine hemocytometer, American Optical, Buffalo, NY, USA), using trypan blue (Invitrogen, Carlsbad, CA, USA). A total of 2×10^5 live cells were pelleted, suspended in 200 μl lysis reagent and incubated for 30 min on ice. The lysate mixture was centrifuged at 16 000 *g* for 20 min at 4 °C, and the supernatant was carefully transferred to a fresh tube. Two microliters of cell extract (corresponding to 2×10^3 cell equivalents) were added to the PCR reaction. Each sample was done in duplicate and had its own separate negative control (heat treated). The human kidney (293) cell line was used as a positive telomerase activity control and standard. Absorbances of the samples were measured at 450 nm (with a reference wavelength of 690 nm) within 30 min after addition of the Stop reagent, using a BioTek PowerWave HT Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Absorbance reading unit (AU) was reported as the A450 nm reading against reference wavelength A690 nm reading (A450 nm – A690 nm unit). Samples were regarded as telomerase positive if ΔA (AU) $= A_{\text{sample}} - A_{\text{negative control}}$ is > 0.2 AU. Intra-assay CV was 5.9% and inter-assay CV was 4.8%. Baseline and 16-week post-test samples of the same participants were measured on the same plate by the same lab investigator who was blinded to all information about the participants.

Statistical analysis

Descriptive statistics are presented as mean ± s.d. if not stated otherwise. Differences in baseline group descriptive characteristics were compared by independent *t*-tests if data were distributed normally. Group differences for categorical variables were tested by Fisher's exact tests. Serum 25(OH)D and PBMC telomerase activity in response to vitamin D supplementation were analyzed using repeated-measures analysis of variance (ANOVA, two groups by two time points). When indicated by significant interaction, simple main effects were performed to identify where the specific differences exist. Age, sex and BMI were considered as potential confounders. Subsequently, conclusions were drawn because all independent variables consisted of only two levels and 1 degree of freedom. All tests were conducted two-sided, and a *P*-value < 0.05 was considered statistically significant. All statistics were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Of the 57 subjects randomized, 8 subjects (4 in each group) were unobtainable/unresponsive to follow-up and were dropped from the study. Eight subjects had PBMCs collected only during the first visit. Additionally, the numbers of PBMC collected from 4 subjects were too low to run the telomeric repeat amplification protocol assay. Therefore, pre and post PBMC telomerase activity testing were measured for remaining 37 subjects (19 subjects in the vitamin D group). Baseline subject characteristics are presented in Table 1. Except for the greater baseline LDL level in the placebo group compared with the vitamin D group, all other subject characteristics at baseline were similar between the two groups.

Response of serum 25(OH)D and PBMC telomerase activity to vitamin D3 supplementation

Serum 25(OH)D levels did not differ between groups at baseline. A significant group × time interaction ($F_{1,36} = 62.8$; $P < 0.0001$) was detected. Serum 25(OH)D level increased significantly from 35.4 ± 11.3 to 103.7 ± 31.5 nmol l^{-1} in the vitamin D group ($F_{1,18} = 86.8$; $P < 0.0001$, Figure 1), whereas it only modestly increased from 40.7 ± 15.7 to 48.1 ± 17.5 nmol l^{-1} in the placebo group (F1,17 = 11.3; $P = 0.004$) at baseline (in February) and after 16 weeks (in May), possibly because of increased sun exposure. These findings also indicated that subjects in the placebo group remained vitamin D insufficient (25(OH)D < 50 nmol l^{-1}) even after statistically significant, although modest, increase in their serum 25(OH)D level at post-testing. No differences in PTH or urinary calcium, either between groups or within groups were observed at either baseline or at posttest (data not shown).

Table 1. Baseline subject characteristics

Variable	*Placebo*	*Vitamin D*	P-*value*
N	18	19	–
Male/female[a]	6/12	7/12	0.593
Age (years)	31.9 ± 10.3	31.1 ± 10.0	0.799
Height (cm)	1.70 ± 0.1	1.70 ± 0.1	0.988
Weight (kg)	87.2 ± 21.9	85.6 ± 25.4	0.846
BMI (kg m^{-2})	30.4 ± 7.7	29.9 ± 8.9	0.844
SBP (mm Hg)	121.2 ± 13.9	124.9 ± 18.4	0.500
DBP (mm Hg)	74.1 ± 10.8	74.8 ± 10.1	0.852
Total cholesterol (mg dl^{-1})	161.7+56.1	135.5 ± 29.5	0.090
HDL (mg dl^{-1})	46.4 ± 15.2	55.2 ± 16.9	0.108
LDL (mg dl^{-1})	121.4 ± 58.0	67.6 ± 28.1	0.006
Triglycerides (mg dl^{-1})	83.5 ± 27.4	72.6 ± 21.6	0.194
HbA1c (%)	5.5 ± 0.5	5.4 ± 0.5	0.553
25(OH)D (nmol l^{-1})	40.7 ± 15.7	35.4 ± 11.3	0.249
Telomerase activity (AU)	1.43 ± 0.26	1.56 ± 0.29	0.146

Abbreviations: AU, absorbance reading unit; BMI, body mass index; DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; HDL, high-density lipo-protein; LDL, low-density lipo-protein; SBP, systolic blood pressure. Values are mean ± s.d. All results not marked were based on independent *t*-test.
[a]Test of significance between the groups were based on Fisher's exact test.

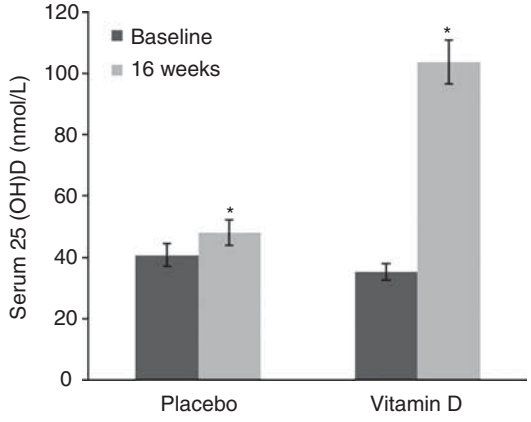

Figure 1. The effect of 16 weeks of placebo or vitamin D3 supplementation on 25 hydroxyvitamin D (25(OH)D) (mean ± s.e.). *Significant from baseline.

PBMC telomerase activity did not differ between the two groups at baseline. A significant group × time interaction ($F_{1,36} = 13.3$; $P = 0.001$) was observed. PBMC telomerase activity increased significantly by 19.2% in the vitamin D group from baseline 1.56 ± 0.29 AU to posttest 1.86 ± 0.42 AU ($F_{1,18} = 20.0$; $P < 0.0001$). The significance persisted after further adjustment for age, sex and BMI ($P = 0.039$). In the placebo group, PBMC telomerase activity remained unchanged from baseline 1.43 ± 0.26 AU to posttest 1.46 ± 0.27 AU ($F_{1,17} = 2.2$; $P = 0.157$) (Figure 2).

We also found significant correlation between the degree of increase in telomerase activity and the serum vitamin D levels after the treatment with each individual even after adjusting with age, sex and BMI ($r = 0.421$, $P = 0.013$).

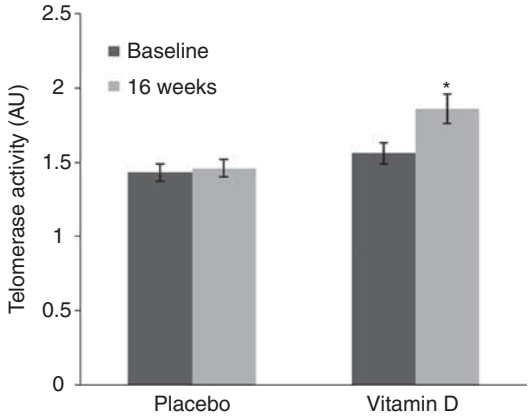

Figure 2. The effect of 16 weeks of placebo or vitamin D3 supplementation on PBMC telomerase activity (mean ± s.e.). *Significant from baseline.

DISCUSSION

We recently conducted a 16-week double blind, randomized clinical trial of vitamin D3 supplementation, placebo vs 60 000 IU monthly (equivalent to 2000 IU per day) in overweight African-American adults.[28] The major finding of the present study is that vitamin D3 supplementation significantly increased PBMC telomerase activity by 19.2% during the time course of 16-week intervention. Our data suggest that vitamin D may improve telomere maintenance and prevent cell senescence.

To our knowledge, our study is the first randomized placebo-controlled clinical trial to evaluate the effect of oral vitamin D3 supplementation on PBMC telomerase activity in humans, particularly in overweight African Americans who are at greater risk for both vitamin D deficiency and cardiovascular disease. The pioneer study conducted by Ornish *et al*.[17] showed that a 3-month life style intervention, including nutrition and physical activity modification, increased PBMC telomerase activity by 29% in 24 low-risk prostate cancer patients. Our randomized clinical trial demonstrated that 16 weeks of 60 000 IU monthly (2000 IU daily) oral vitamin D3 supplementation was able to increase PBMC telomerase activity by 19.2% in overweight African-American adults. Our findings support the previously reported cross-sectional observation that higher serum 25(OH)D level is associated with longer leukocyte telomere length,[29] which may be achieved by increased telomerase activity. The anti-cellular aging function of vitamin D might result from its anti-inflammation, anti-oxidative stress effects[32-34] or through nongenomic activation of a VDR/PI3K/Akt survival pathway.[35-38]

PBMCs are critical components in the immune system involved in cytotoxicity and the production of cytokines and chemokines. Telomerase activity is an important determinant of telomere length and may be an earlier predictor of long-term cellular viability or genomic stability than telomere length.[17] Increasing telomerase activity not only affects the telomeres and proliferative potential but also preserves healthy cell function and long-term immune function.[7,8] Emerging evidence show that telomerase contributes to cell physiology independently of its ability to elongate telomeres. Telomerase promotes proliferation of resting stem cells,[39] functions as a transcriptional co-activator in Wnt signaling,[40] and regulates mitochondrial function.[41] Telomerase offers cell protection especially in the context of shorter telomeres.[42] Obese individuals tend to have shorter telomeres.[1-4] The increase in PBMC telomerase activity shown in our study suggests that vitamin D supplementation could help to counteract obesity-induced acceleration of immune cellular aging and improve immune cell function.

Vitamin D has known immunomodulatory effects on a wide range of immune cells, including $CD4^+$ and $CD8^+$ lymphocytes, B lymphocytes, macrophages as well as dendritic cells, which are the main components of PBMCs.[18-43] Several *in vitro* studies revealed that $1,25(OH)_2D_3$ increases regulatory $CD4^+$ T-cell percentages,[44-46] and may reduce $CD8^+$ T-cell percentage.[47] Moreover, there are differences in telomerase dynamics between CD4 and CD8 T cells.[7-48] Therefore, we cannot exclude the

possibility that vitamin D-induced increase in PBMC telomerase activity is due to the increase in certain cell types within the PBMC with higher telomerase activity, as we did not sort the subtypes of PBMC at the time of collection and telomerase measurement. Future studies to clarify the issue are warranted.

Vitamin D supplementation has been shown to have many positive health benefits including on cardiovascular health.[49,50] We recently conducted two 16-week randomized clinical trials of 2000 IU vitamin D3 supplementation daily, and showed an improved arterial stiffness in African-American adolescents,[27] and an improved endothelial function in overweight African-American adults.[28] These results suggest that vitamin D might decelerate the vascular aging process. Recently, Jaskelioff *et al*.[51] showed that in aged, telomerase-deficient mice, telomerase reactivation can reverse tissue degeneration. The anti-cellular aging effect of vitamin D in overweight African Americans demonstrated in the present study adds to the growing list of beneficial effects of vitamin D.

We observed significant difference in LDL levels between placebo and vitamin D groups at the baseline. We acknowledge that high LDL might influence leukocyte telomerase activity to a lesser extent by increasing the amount of oxidized LDL in serum and thus, affecting cell growth and cytokine production. Tsirpanlis *et al*.[52] found an inverse correlation between oxidized LDL and PBMC telomerase activity in hemodialysis patients (mean age 51 years) living in Athens, Greece. However, we did not find any significant correlation between LDL and telomerase activity both at the baseline and after treatment in our young and relatively healthy African-American study population. Further adjustment for LDL along with age, sex, and BMI did not change the result.

The major strengths of the present study include: (1) a randomized, double-blinded and placebo-controlled clinical trial can overcome the problem of confounding, which exists in observational studies; (2) 100% compliance achieved by investigator-supervised dosing helps to overcome the major disadvantage in clinical trials.

However, the limitations and concerns are noteworthy. First, the sample size of this study is relatively small. Therefore, our findings warrant replication in a larger study. Second, our results were observed following a 16-week supplementation, longer-term clinical trials (for example, 12 months) are warranted to demonstrate the sustainability of the effects of vitamin D3 supplementation on telomerase activity and telomere length. Third, we did not sort subtypes of PBMC at the time of sample collection. Therefore, we cannot exclude the possibility that vitamin D-induced increase in PBMC telomerase activity is due to the increase in certain cell types within the PBMC with higher telomerase activity. Fourth, whether our data obtained in African-American adults are generalizable to other population remains to be determined. Last, the underlying cellular mechanisms of our findings need to be further investigated.

In conclusion, for the first time we have demonstrated that vitamin D supplementation increased PBMC telomerase activity in overweight African Americans, suggesting that vitamin D may have an anti-cellular aging/senescence property. Large independent clinical trials with a longer-term vitamin D supplementation with different dose and across different racial/ethnic groups are warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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