Supplementary Data

Effects of vitamin D and L-cysteine cosupplementation on circulating bioavailable and total 25-hydroxy-vitamin D, the free/total testosterone ratio and inflammatory biomarkers in healthy vitamin D-deficient African Americans: a placebo-controlled double-blind clinical trial

Trial Eligibility

Our study began with a sample size of 257 consented participants, of whom 165 participants met the eligibility requirements and were enrolled successfully (Fig. 1). Upon enrollment, all participants agreed to complete health history questionnaires and basic health examinations to confirm a good general health history. In addition, participants agreed to stop taking vitamin D, probiotics, multivitamins, or any other supplements not prescribed to them by investigators, due to their potential interference with study results.

Consisting of locating and educating prospective participants on the purpose and design of the clinical trial, participants were approached and selected at random. To prevent bias, prospects were selected from a variety of different areas and backgrounds. Protocols established by the Internal Review Board (IRB) were closely scrutinized to ensure that no disadvantaged persons felt coerced to join the trial due to financial incentives. Enrollment and recruiting were conducted consistently by the investigative team until the participant goal was reached. Subjects who agreed to participate were given the following materials: informed consent forms that explained the purpose and outlined the responsibilities of the investigative team, as well as the consented subjects; a demographics form to capture information such as age, sex, occupation, allergies, and pre-existing medical conditions; and HIPAA forms that informed subjects of their right to privacy and assured them that all privileged health information would remain confidential.

Exclusion criteria

Those excluded from the study were subjects who had taken dietary supplements within the 2 months previous to enrollment or during the study; any subject who developed an infection and had to take antibiotics; women who were nursing; or any subject taking vitamin D or any other vitamin or herbal supplement, even when advised to do so by a doctor, or any over-the-counter supplement. With each clinical visit, women of childbearing age were required to submit to a pregnancy test. Negative test results were required to continue participation. Women over 60 years of age with at least a year without a menstrual cycle, or women who had had either a

hysterectomy or tubal ligation, were exempt from the pregnancy tests. Participants who failed to meet or comply with the inclusion and exclusion criteria were excluded from the study.

Compliance was ensured regularly through weekly telephone calls from the investigator and the study coordinator. Unused capsules from the previous visits were counted and recorded for all subjects. The use of any unprescribed supplements and other concerns were documented at each visit. None of the participants reported any side effects during the study period.

ELISA (enzyme-linked immunosorbent assay)

Plasma levels of VDBP (ALPCO, Diagnostic, Salem, NH); insulin, SHBG, CRP, total testosterone (R&D Systems, Minneapolis, MN); and PTH and free testosterone (abcam, Cambridge, UK) were determined using ELISA kits. All appropriate controls and standards as specified by the manufacturer's kits were used. Control samples were assayed each time to check the variations between the plates on different days to reduce the variability of assays. All the assays were done in duplicate for each sample and at the same time for both baseline and final visit samples. The concentration of each assay was calculated using a standard curve with appropriate blanks.

RNA extraction and qRT-PCR

The concentration and quality of the isolated RNA were measured on a NanoDrop spectrophotometer (Thermo Scientific, Swedesboro, NJ). cDNA was synthesized from 1 µg of RNA using a High-Capacity RNA-to-cDNA Kit (Applied Biosystem, Waltham, MA) in a 20 µL reaction volume and then incubated at 37 °C for 60 min followed by 95 °C for 5 min. Quantitative PCR was performed using RT^2 SYBR Green (QIAGEN, Germany) in an Opus 384-2 Real-Time System (Bio-Rad, Hercules, CA). Gene-specific primers were designed using PrimerBlast software and synthesized by Invitrogen (Table S1). Melting curve analysis was performed each time to check the specificity of primers. Each sample was run in triplicate and the relative expression (ΔΔt) of the mRNA was calculated and normalized to the housekeeping gene *GAPDH*.

Table S1. List of primers included in Quantitative PCR.

Gene	Orientation	Primer sequence
CYP2R1	Forward	GAGAGACACAAAGGTGCAAATG
	Reverse	TGGAGTCAAGAAGGGATGAAAG
CYP27A1	Forward	CGTCAGATCCATCGGGTTAATG
	Reverse	CATTCCAACCATCCAGGTATCG
CYP27B1	Forward	TGGCCCAGATCCTAACACATTT
	Reverse	GTCCGGGTCTTGGGTCTAACT

VDR	Forward	CCTTCACCATGGACGACATG
	Reverse	CGGCTTTGGTCACGTCACT
SHBG	Forward	CCTGAACAGAAGCCATGAGAT
	Reverse	AGGTGGAGCTTTAATGGGAAG
CYP11A1	Forward	CGTCAGATCCATCGGGTTAATG
	Reverse	CATTCCAACCATCCAGGTATCG
CYP19A1	Forward	GAGAACCAGGCTACAAGAGAAA
	Reverse	TGGTGGAATCGGGTCTTTATG
GAPDH	Forward	AGTATGACAACAGCCTCAAGAT
	Reverse	GTCCTTCCACGATACCAAA

Table S2. Baseline and 6-month post-treatment characteristics for women compared among the treatment groups. Data presented as mean±SE.

Characteristic	Placebo (n=18)		L-cysteine 1000 IU (n=24)		Vitamin D 2000 IU (n=17)		VD+LC (n=16)	
	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment
Age (years)	41.9±3.1	41.9±3.1	43.7±2.5	43.7±2.5	45.8±3.3	45.8±3.3	42.8±3.7	42.8±3.7
BMI (kg/m^2)	36.1±2	36.5±2	32.9±1.4	33.5±1.5	34.1±2	34.6±1.9	34.2±2.5	33.7±2.3
FIB-4 (score)	0.63±0.1	0.64±0.07	0.51±0.04	0.60±0.06	0.7±0.1	0.7±0.1	0.54±0.07	0.57±0.08
Glucose (mg/dL)	91.7±3.8	95±2.7	90.2±2.2	96.1±2.3	102.5±4.2	102±3.7	91±2.3	92.9±2.5
Creatinine (mg/dL)	0.87±0.04	0.87±0.03	0.83±0.03	0.9±0.04	0.8±0.02	0.8±0.03	0.8±0.02	0.8±0.03
Calcium (mg/dL)	9.4±0.1	9.2±0.08	9.4±0.05	9.2±0.07	9.3±0.1	9.3±0.09	9.3±0.09	9.2±0.13
Alkaline phosphatase (IU/L)	74±5.5	72.8±5.3	85.1±6.4	84±7.3	80.7±5.9	80±4.9	73±5.7	75.3±6.2
ALT (IU/L)	23.4±1.5	23.9±2	22.2±1.2	24.4±2	24.7±2.3	23.8±2.4	23.7±2.2	25.9±5.5
AST (IU/L)	18.1±1.7	19.6±1.5	15.9±0.8	19.4±1.5	17.9±1.9	17.9±1.2	17.9±1.6	20.2±3.7
Total 25(OH)VD (ng/mL)	17.6±1.6	19.7±1.3	16.6±1.2	18.6±1.5	17.2±1.8*	31.9±3.8$^{\#}$	17.5±2**	27.7±2.9$^{\#\#}$
Free 25(OH)VD (ng/mL)	4.3±0.8	2.5±0.36	2.4±0.47	2.47±0.28	3.4±0.7	3.5±0.57	4±0.74	3.32±0.7
Bioavailable 25(OH)VD	3.8±0.54	4.17±0.4	3.8±0.3	4.35±0.4	4.3±0.5	3.8±0.57$^{\wedge}$	3.3±0.4$^{\wedge\wedge}$	5±0.7$^{\wedge\wedge\wedge}$
Hb (g/dL)	12.8±0.3	12.4±0.3	12.8±0.2	12.4±0.3	13±0.2	12.6±0.2	12.3±0.3	12±0.34
PTH (pg/mL)	32.8±4	29.3±2.3	27.6±2.4	24.7±2	34±4.5*	28.4±3.7$^{\#}$	31.3±4.6	25±2.7

*vs#, **vs##, ^vs^^, ^vs^^^ p≤0.05; FIB-4, Fibrosis-4 index; ALT, alanine transaminase; AST, aspartate transaminase; Hb, hemoglobin; PTH, parathyroid hormone.

Table S3. Baseline and 6-month post-treatment characteristics for men compared among the treatment groups. Data presented as mean±SE.

Characteristic	Placebo (n=4)		L-cysteine 1000 IU (n=6)		Vitamin D 2000 IU (n=6)		VD+LC (n=5)	
	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment
Age (years)	34.5±6.6	34.5±6.6	39.8±4.8	39.8±4.8	34.8±4.7	34.8±4.7	31.2±5	31.2±5
BMI (kg/m^2)	37.6±8	37±7.5	32.4±1.7	34±2.2	29.9±4.4	29.7±4.2	26±1.9	27±1.6
FIB-4 (score)	0.8±0.2	0.8±0.2	0.7±0.1	0.7±0.2	0.5±0.1	0.5±0.07	0.5±0.05	0.6±0.2
Glucose (mg/dL)	88.8±3	92±0.9	101±6.6	106±9.6	89.3±5.4	88.3±2.2	92±7.4	96±4
Creatinine (mg/dL)	1±0.09	1±0.1	1±0.03	1±0.04	1±0.03	1±0.03	1±0.06	1±0.05
Calcium (mg/dL)	9±0.1	9±0.2	9±0.2	9±0.2	9.5±0.2	9.3±0.2	9.6±0.2	9±0.2
Alkaline phosphatase (IU/L)	76±8.7	70±6.5	73.8±5	72.8±6.9	65.3±6.3	66.2±7.8	66±9.7	56±7.3
ALT (IU/L)	32.8±5.8	32.5±4.7	36.7±4.7	32.2±2.4	29.2±4	25±3.5	37±7	40.4±6.3
AST (IU/L)	26.8±3.4	28.3±4.3	23.7±1.9	23.3±3	19.3±2.2	18.3±1.3	28.2±3.2	24±4
Total 25(OH)VD (ng/mL)	20.5±3.4	30.5±5	12.8±1.3	16±1.6	16.3±1.7*	21±3$^{\#}$	12.8±0.8**	33±2$^{\#\#}$
Free 25(OH)VD (ng/mL)	4.19±1.95	5.24±0.67	1.48±0.16	2.34±0.21	3.53±1.2	3.85±0.96	2.7±0.83	3.2±0.47
Bioavailable 25(OH)VD	4.65±1.4	5.07±2.13	3.46±0.3	5.42±0.4	3.63±0.9	4.29±1.16$^{\wedge}$	4.12±0.8$^{\wedge\wedge}$	5.51±1.42$^{\wedge\wedge\wedge}$
Hb (g/dL)	14.7±0.3	14±0.3	14.3±0.5	13.9±0.2	14.5±0.4	14.2±0.4	14.9±0.3	15±0.4
PTH (pg/mL)	22.4±4.5	22.4±6.8	28.7±6	20.9±6.4	18.4±2.6*	20.4±4$^{\#}$	24.5±6	24±6

*vs#, **vs##, #vs##, ^vs^^^, ^^vs^^^ p≤0.05; FIB-4, Fibrosis-4 index; ALT, alanine transaminase; AST, aspartate transaminase; Hb, hemoglobin; PTH, parathyroid hormone.

Table S4. Characteristics of individuals with a BMI≤30 compared among the treatment groups (men and women) at baseline and after 6 months of treatment. Data presented as mean±SE.

Characteristic	Placebo (n=7)		L-cysteine 1000 IU (n=11)		Vitamin D 2000 IU (n=12)		VD+LC (n=12)	
	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment
Age (years)	37.29±6.2	37.29±6.2	39.18±3.93	39.18±3.93	37.92±4.54	37.92±4.54	38.25±3.82	38.25±3.82
BMI (kg/m^2)	26.74±0.84	27.09±0.7	26.18±0.88	26.30±0.82	25.68±0.82	26.33±0.79	25.83±0.96	26.23±1.01
FIB-4 (score)	0.59±0.07	0.71±0.1	0.45±0.05	0.53±0.06	0.54±0.1	0.57±0.1	0.6±0.08	0.6±0.1
Glucose (mg/dL)	88.43±3.26	94.57±1.97	92.36±3.54	90.09±2.53	92.25±3.24	93.3±2.63	88.75±2.84	90.75±2.42
Creatinine (mg/dL)	0.95±0.1	0.95±0.1	0.78±0.05	0.83±0.04	0.87±0.04	0.91±0.06	0.88±0.05	0.86±0.05
Calcium (mg/dL)	9.31±0.16	9.1±0.1	9.39±0.11	9.14±0.13	9.47±0.1	9.23±0.07	9.34±0.1	9.12±0.1
Alkaline phosphatase (IU/L)	73.14±9.23	74.43±9.18	70±8.29	69.55±9.15	72.5±6.37	70.58±5.49	57±4.05	59.92±5.52
ALT (IU/L)	27±4.15	27.71±3.47	23.91±3.56	21.09±2.17	25.67±2.37	22.83±2.33	26.5±3.84	34±7.56
AST (IU/L)	19.29±3	21.57±3.57	15.55±1.13	17.64±1.37	18.67±2.26	17.83±1.56	21.83±2.7	23.33±4.8
Total 25(OH)VD (ng/mL)	19.33±2.92	26.36±3.62	17.15±1.55	17.79±2	17.13±2*	33.86±4.69$^{\#}$	15.73±2.07**	28.19±2.71$^{\#\#}$
Free 25(OH)VD (ng/mL)	5.79±1.35	3.76±0.9	1.51±0.09	2.85±0.48	3.61±0.87	3.73±0.74	3.85±0.81	3.11±0.44
Bioavailable 25(OH)VD	3.56±0.8	4.45±0.9	3.59±0.2	4.22±0.58	4.08±0.56	3.74±0.57	3.81±0.56	4.7±0.91
Hb (g/dL)	13.74±0.55	13.33±0.6	13.17±0.34	12.59±0.34	13.5±0.37	12.98±0.35	13.38±0.39	13.31±0.44
PTH (pg/mL)	23.95±4.37	22.14±2.95	21.91±3.58	21.29±2.63	25.84±3.57	23.02±3.48	26.7±5.13	25.18±3.28

*vs#, **vs##, #vs## p≤0.05; FIB-4, Fibrosis-4 index; ALT, alanine transaminase; AST, aspartate transaminase; Hb, hemoglobin; PTH, parathyroid hormone.

Table S5. Characteristics of individuals with a BMI>30 compared among the treatment groups (men and women) at baseline and after 6 months of treatment. Data presented as mean±SE.

Characteristic	Placebo (n=15)		L-cysteine 1000 IU (n=19)		Vitamin D 2000 IU (n=11)		VD+LC (n=9)	
	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment
Age (years)	42.07±3.07	42.07±3.07	45.11±2.51	45.11±2.51	48.36±2.62	48.36±2.62	42.44±5.55	42.44±5.55
BMI (kg/m^2)	40.83±2.2	41.08±2.07	36.67±0.96	37.85±1.05	40.99±1.9	40.97±1.8	40.89±2.7	39.98±2.32
FIB-4 (score)	0.68±0.13	0.64±0.09	0.59±0.06	0.67±0.08	0.72±0.19	0.72±0.14	0.47±0.07	0.55±0.14
Glucose (mg/dL)	92.4±4.36	94.27±3.21	92.26±2.97	102.63±3.5	106.55±5.88	104.1±5.29	94.56±3.93	97.56±3.53
Creatinine (mg/dL)	0.89±0.04	0.92±0.04	0.92±0.03	0.95±0.04	0.84±0.04	0.87±0.04	0.83±0.04	0.87±0.04
Calcium (mg/dL)	9.35±0.1	9.17±0.1	9.26±0.06	9.19±0.08	9.26±0.15	9.28±0.14	9.43±0.14	9.21±0.23
Alkaline phosphatase (IU/L)	74.67±5.56	71.33±5.16	90.32±6.32	88.79±7.6	81.18±7.31	82.91±6.22	90.56±5.09	84.89±7.78
ALT (IU/L)	24.2±1.69	24.47±2.32	25.79±1.77	28.79±2.27	26.09±3.45	25.55±3.31	27.11±3.27	23.11±3
AST (IU/L)	19.87±2.09	20.93±1.75	18.53±1.26	21.68±1.97	17.91±2.1	18.27±1.1	18.44±1.54	17.89±2.34
Total 25(OH)VD (ng/mL)	17.51±1.68	19.53±1.4	15.36±1.38	18.21±1.65	17.58±2.05*	26.51±3.15$^{\#}$	17.22±2.65**	29.93±4.19$^{\#\#}$
Free 25(OH)VD (ng/mL)	3.6±0.8	2.65±0.38	2.62±0.59	2.19±0.21	3.2±0.83	3.45±0.61	3.53±0.96	3.55±0.74
Bioavailable 25(OH)VD	4.15±0.64	4.27±0.6	3.78±0.42	4.78±0.48	4.12±0.73	4.12±0.91	3.09±0.49	5.70±0.8
Hb (g/dL)	12.84±0.38	12.44±0.32	12.97±0.29	12.75±0.32	13.24±0.27	13±0.28	12.24±0.6	12.01±0.63
PTH (pg/mL)	34.17±4.48	30.77±2.71	31.27±2.64	25.48±2.71	34.34±6.62	30.10±4.99	33.59±5.57	24.7±3.51

*vs#, **vs## p≤0.05; FIB-4, Fibrosis-4 index; ALT, alanine transaminase; AST, aspartate transaminase; Hb, hemoglobin; PTH, parathyroid hormone.