

The Human Skeletal Muscle Transcriptome in Response to Oral Shilajit Supplementation

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ABSTRACT The objective of the present study (clinicaltrials.gov NCT02026414) was to observe the effects of oral supplementation of a purified and standardized Shilajit extract on skeletal muscle adaptation in adult overweight/class I obese human subjects from the U.S. population. Shilajit is a mineral pitch that oozes out of Himalayan rocks. The study design consisted of a baseline visit, followed by 8 weeks of 250 mg of oral Shilajit supplementation b.i.d., and additional 4 weeks of supplementation with exercise. At each visit, blood samples and muscle biopsies were collected for further analysis. Supplementation was well tolerated without any changes in blood glucose levels and lipid profile after 8 weeks of oral supplementation and the additional 4 weeks of oral supplementation with exercise. In addition, no changes were noted in creatine kinase and serum myoglobin levels after 8 weeks of oral supplementation and the additional 4 weeks of supplementation with exercise. Microarray analysis identified a cluster of 17 extracellular matrix (ECM)-related probe sets that were significantly upregulated in muscles following 8 weeks of oral supplementation compared with the expression at the baseline visit. This cluster included tenascin XB, decorin, myoferlin, collagen, elastin, fibrillin 1, and fibronectin 1. The differential expression of these genes was confirmed using quantitative real-time polymerase chain reaction (RT-PCR). The study provided maiden evidence that oral Shilajit supplementation in adult overweight/class I obese human subjects promoted skeletal muscle adaptation through upregulation of ECM-related genes that control muscle mechanotransduction properties, elasticity, repair, and regeneration.

KEY WORDS: • adaptation • extracellular matrix • Shilajit • skeletal muscle

INTRODUCTION

IN RECENT YEARS, understanding effectiveness of nutritional supplements in enhancing skeletal muscle performance and attenuating muscle injury has gained marked interest.¹ Shilajit is a mineral pitch that seeps out of the rocks in the high altitudes of Himalayan Mountains.² In traditional Ayurvedic medicine, Shilajit has been reported to exhibit adaptogenic and potent anabolic properties.^{3,4} Furthermore, Shilajit has been used for centuries to treat a number of disorders, including muscle and tendon injuries.^{2,5} A derivative of Shilajit, fulvic acid (FA) complex, consists of naturally occurring low- and medium-molecular-weight

compounds, including oxygenated dibenzo-alpha-pyrones (DBPs) and acylated DBPs.^{5,6} In albino mice, Shilajit supplementation significantly enhanced physiological energy status in a forced swimming test model.⁷ DBP, FA, and their derivatives are the principal constituents of Shilajit contributing to these effects.^{4,8}

The skeletal muscle is made up of heterogeneous muscle fibers with distinct metabolic and contractile properties.⁹ Changes in gene expression is an integral component of skeletal muscle physiological adaptations to exercise and nutritional supplementation.⁹ Shilajit has been reported to improve physical performance and relieve fatigue with enhanced adenosine triphosphate (ATP) production.^{4,8,10} The primary aim of the present longitudinal study (the same person's initial visit serves as the baseline for subsequent visits) is to find out the effect of oral Shilajit supplementation and exercise training on human skeletal muscle adaptation in a group of overweight/class I obese human subjects following 12 weeks of study period. This particular cohort was selected for the study because physiological muscle performance is often compromised in this group of subjects.^{11–13}

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MATERIALS AND METHODS

Natreon Inc.'s patented ingredient, PrimaVie® [US 6,969,612; 6,440,436; 6,558,712; 8,894,993; and EP 1 387 614], is a purified and standardized Shilajit extract for nutraceutical use. It is standardized to have not less than 60.3% fulvic acid equivalents with DBP and associated chromoproteins (50% fulvic acid + 10.3% free DBPs and DBPs conjugated with chromoproteins) in Shilajit extract. The test product, PrimaVie Shilajit (PVS) capsules, 250 mg, was supplied by Natreon, Inc., New Brunswick, NJ, USA. PVS is manufactured by a process to reduce the heavy metals to less than 1 ppm of lead, 1 ppm of arsenic, and less than 0.1 ppm of mercury. Quality control is achieved through high-performance liquid chromatography (HPLC) analysis as previously reported.¹⁴

Ethics, consent, and permissions

The Western Institutional Review Board (WIRB) approved the study protocols (clinicaltrials.gov NCT02026414) and materials. All subjects provided written informed consent before participation in the study.

Study subjects and experimental design

Overweight/Class I obese, adult human subjects (21–70 years) of both genders with body-mass index (BMI) 25–35 were entitled to participate in this study. They were asked to fast overnight, following which collection of blood samples was done. Any self-reported variations in diet or exercises were recorded. The subjects were excluded from the study if any one of the following medications was used for management/treatment of cardiovascular disease (CVD)-related disorders: steroids (Prednisone, etc.), beta-blockers, hydrochlorothiazide, statins (Crestor, Lipitor, etc.), aspirin, and angiotensin-converting enzyme (ACE) inhibitors. Pregnant females as well as individuals who were therapeutically immunocompromised were also excluded from the study. The experimental study design consisted of four study visits during the 12-week study period: *visit 1*, baseline visit; *visit 2*, after 8 weeks of oral supplementation of PVS; *visit 3a*, additional (following 8 weeks of initial supplementation) 4 weeks of oral supplementation and exercise, sample collection before the final bout of exercise; and *visit 3b*, same as study visit 3, sample collected 30 min post-final bout of exercise. At each study visit, 50 mL of blood, 5 mm muscle biopsy, and demographic information, including age, gender, weight, BMI, blood pressure, and pulse, were taken (Table 1).

Supplementation regimen and compliance

Each subject received 250 mg of PVS capsules twice a day for the first 8 weeks of study period. For the last 4 weeks of the study, subjects took 250 mg of oral PVS supplement twice a day while also completing exercise on a treadmill (70–75% of maximum heart rate for 20 min monitored using Polar FT4, plus 5 min of warm-up and 5 min of cool-down exercises for a total of 30 min a day, 3 days a week). The same exercise regimen was followed during visits 3a and 3b.

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF STUDY PARTICIPANTS

Parameters	Values	Baseline	8 Weeks	12 Weeks
Subjects (<i>n</i>)	16			
Age (years)	35.7 ± 3.4			
Gender				
Males	6			
Females	10			
Body weight (lb.)		188.5 ± 8.6	187.9 ± 8.8	187.4 ± 8.9
Body-mass index, kg/m ²		28.9 ± 0.6	28.8 ± 0.7	29.3 ± 0.7
Blood pressure				
systolic, mmHg		114.3 ± 3.1	114.8 ± 2.8	112.1 ± 2.5
Blood pressure				
diastolic, mmHg		74 ± 1.9	71.6 ± 1.5	69.8 ± 1.8
Pulse (min)		70.1 ± 2.0	72.4 ± 1.9	70.8 ± 2.2

Values are expressed as mean ± SEM.

All subjects participated in the total 12 weeks of study, including one baseline and three follow-up visits. The dose of PVS was chosen based on an earlier human supplementation study.¹⁴ The 8-week period of supplementation was selected based on earlier studies on biochemical/genetic adaptations in skeletal muscles.^{15,16}

Safety monitoring

No adverse effect directly related to the dietary supplement was reported by clinical research staff.

Blood sampling and analysis

During each visit, peripheral venous blood was collected in heparinized tubes and transported on ice immediately for analysis. Among lipid profile total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride levels, calculated LDL-C, and non-HDL-C were measured using standard clinical lipid profile,¹⁷ and creatine kinase (CK), glucose, and serum myoglobin were measured at the clinical laboratory of Ohio State University Wexner Medical Center.

Muscle biopsy collection

A biopsy was collected by a board-certified physician after application of local anesthetics to the site of biopsy (vastus lateralis) using a 100–120 V, 50–60 Hz, 600VA biopsy machine having 12-gauge SenoRx, stereotactic ultrasound Encor Probe (BARD Encor Ultra, breast biopsy system, Tempe, AZ, USA). Muscle samples were stored in liquid nitrogen for further analysis.

Affymetrix GeneChip® probe array analysis

GeneChip® probe array analysis was performed on RNA extracted from muscle biopsies collected on baseline (visit 1) and visit 2 (8 weeks post-supplementation) using Affymetrix GeneChip Human Transcriptome Array 2.0 as described previously.^{18,19} Briefly, total RNA was isolated and

TABLE 2. LIPIDS, GLUCOSE, AND MUSCLE DAMAGE MARKERS OF BASELINE, 8 WEEKS, AND 9–12 WEEKS (PRE AND POST-FINAL EXERCISE)

Variables	Baseline			8 Weeks			9–12 Weeks (pre-final exercise)			9–12 Weeks (post-final exercise)		
	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM
Creatine kinase (U/L) (normal value: 30–220)	16	121.50	20.12	16	92.13	9.64	16	118.69	18.46	16	142.19	20.28
Glucose (mg/dL) (normal value: 70–99)	16	79.56	2.76	16	80.88	3.00	16	77.50	3.88	16	82.56	2.88
Cholesterol (mg/dL) (normal value: <200)	16	175.06	7.12	16	187.38	8.61	16	184.00	8.65	16	184.50	8.26
Triglycerides (mg/dL) (normal value: <150)	16	137.44	23.74	16	144.88	19.24	16	137.56	27.82	16	143.75	24.03
HDL cholesterol (mg/dL) (normal value: >60)	16	52.06	3.05	16	52.00	2.79	16	55.44	3.80	16	55.31	3.67
LDL cholesterol (calculated) (mg/dL) (normal value: <100; optimal)	16	94.86	8.17	16	105.27	8.45	16	105.87	7.50	16	101.13	7.19
Total cholesterol/HDL (ratio) (normal value: <4.5; low risk)	16	3.51	0.23	16	3.74	0.24	16	3.46	0.21	16	3.47	0.20
Non-HDL cholesterol (mg/dL) (normal value: <130)	16	123.00	7.64	16	135.38	8.67	16	128.56	7.56	16	129.19	7.25
Myoglobin (mcg/L) (normal value: ≤90)	16	40.38	3.02	16	40.06	2.47	15	42.40	3.26	15	66.13	6.90

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

RNA integrity was interrogated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Total RNA was linearly amplified, labeled, and fragmented using the GeneChip WT PLUS reagent kit (Affymetrix, Santa Clara, CA, USA). Labeled cRNA targets hybridized to Affymetrix GeneChip Human Transcriptome Array 2.0 (HTA 2.0) for 16 h at 45°C in hybridization oven (Affymetrix model 640) rotating at 60 rpm were washed, stained, and scanned in our own facilities as described earlier.^{18,19} The expression data have been submitted to the Gene Expression Omnibus (GEO) at NCBI (www.ncbi.nlm.nih.gov/geo/) with the series accession number GSE71219. GCOS (Gene Chip Operating Software; Affymetrix) was employed for data acquisition and image processing. Raw data were analyzed using Genespring GX (Agilent, Santa Clara, CA, USA). Additional processing of data was performed using dChip software (Harvard University).^{18,19} Arrays were normalized using RMA algorithm in Expression Console and comparisons made in Transcriptome Analysis Console (Affymetrix). Differentially expressed genes were identified using a two-class paired *t*-test (visit 1 versus visit 2) where the significance level was set at $P < .05$ with correction for false discovery rate.²⁰ The genes that were significantly upregulated were subjected to functional analysis using DAVID (Database for Annotation, Visualization and Integrated Discovery, NIAID, NIH). Gene ontology (GO) was used to identify broadly gene annotation categories under molecular function and biological process.

Quantification of mRNA expression by real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was carried out to validate the extracellular matrix (ECM)-related genes identified using GeneChip probe array analysis. Real-time polymerase chain reaction (RT-PCR) was performed using double-stranded DNA binding dye SYBR Green-I as described previously.^{21,22} *GAPDH* was used as a reference housekeeping gene.

Statistical methods

Multivariate linear regression was used to test if all 11 gene expression ($\Delta\Delta CT$) values were jointly different across adjacent time points. Five comparisons were generated across various time points. The multivariate regression produces estimated differences along with their 95% confidence interval for each gene with a single *P*-value testing if all 11 $\Delta\Delta CT$ values of the genes were jointly different across adjacent time points. Multivariate normality was checked using standardized normal probability plots. If any values were not normal, then they were transformed using natural logarithms. A new multivariate linear regression model was used to check if patient lipids/glucose/muscle damage marker values were jointly different across adjacent time points. Lipids/glucose/muscle damage marker values were summarized using means and standard deviations for

TABLE 3. MULTIVARIATE TEST OF ALL LIPIDS/GLUCOSE/MUSCLE DAMAGE MARKERS (JOINTLY)

Comparison	Contrast	Standard Error	Z	P
8 weeks versus baseline	-14.01	16.64	-0.84	.400
9–12 weeks (pre-final exercise) versus baseline	-9.78	17.20	-0.57	.570
9–12 weeks (pre-final exercise) versus 8 weeks	4.43	16.94	0.25	.803
9–12 weeks (post-final exercise) versus 8 weeks	26.80	16.94	1.58	.114
9–12 weeks (pre-final exercise) versus 9–12 weeks (post-final exercise)	22.57	17.49	1.29	.197

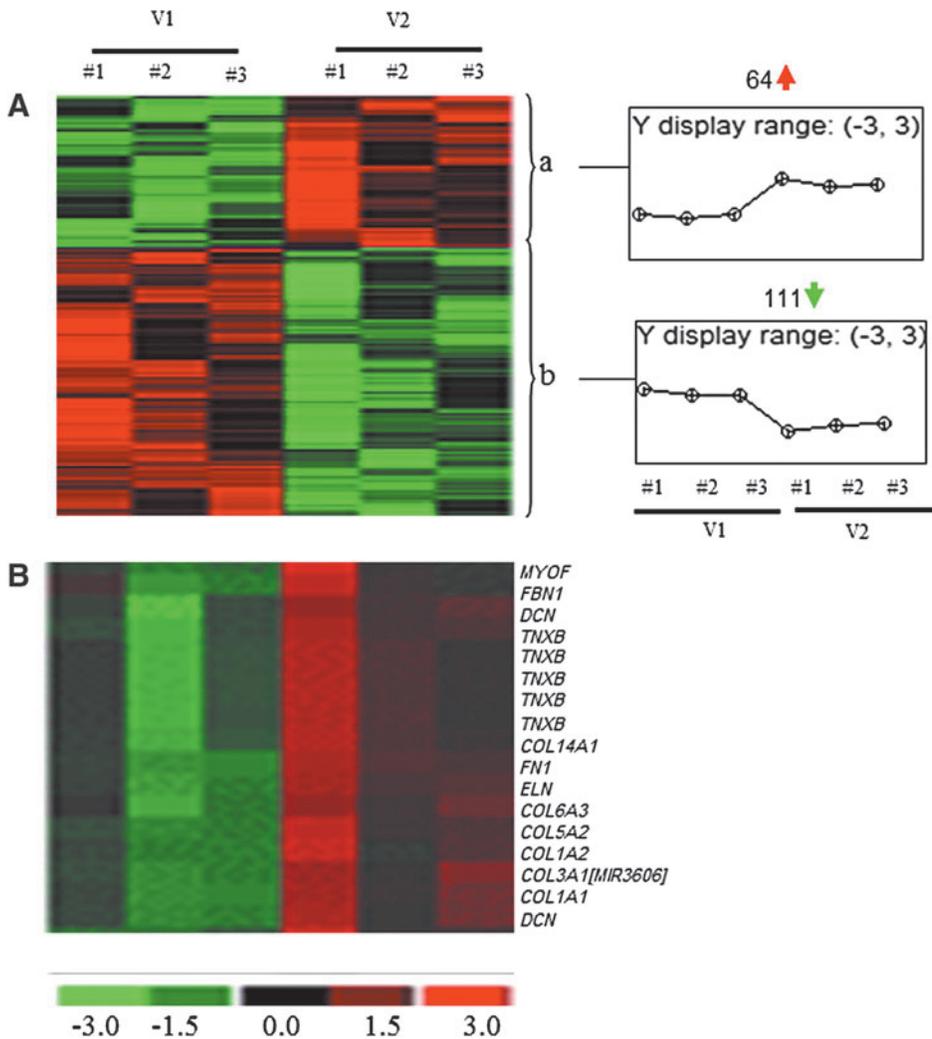


FIG. 1. Heat map illustrating cluster of transcripts that were sensitive to PVS supplementation. PVS-sensitive transcripts were subjected to hierarchical clustering. **(A)** A total of 175 annotated probe sets were differentially ($P < .05$) regulated following 8 weeks of oral PVS supplementation compared with baseline visits. **(B)** Pathway analysis revealed an ECM-related cluster of probe sets that was significantly upregulated following 8 weeks of supplementation compared with corresponding baseline visit. *COL1A1*, collagen type I alpha 1; *COL1A2*, collagen type I alpha 2; *COL5A2*, collagen type V alpha 2; *COL6A3*, collagen type VI alpha 3; *COL14A1*, collagen type XIV alpha 1, *DCN*, decorin; ECM, extracellular matrix; *ELN*, elastin; *FBN1*, fibrillin 1; *FNI*, fibronectin 1; *MYOF*, myoferlin; *TNXB*, tenascin XB; V1, baseline visit; V2, 8 weeks of oral PVS supplementation.

each of the three time points. All analyses were run using Stata 13.1; StataCorp, College Station, TX, USA.

RESULTS

Analysis of glucose, lipid profile, CK, and serum myoglobin levels following oral PVS supplementation

Lipid profile measurements displayed no significant changes in the cholesterol, HDL-C, calculated LDL-C, total cholesterol/HDL, non-HDL-C, and triglycerides following 8 weeks of oral PVS supplementation compared with the baseline levels, suggesting the supplementation was well tolerated (Tables 2 and 3). Additionally, lipid profile levels at the week 12 pre and post-final exercise (visits 3a and 3b) showed no significant changes compared with the baseline levels at 8 weeks (Tables 2 and 3). Moreover, no changes were observed in other variables, including blood glucose and muscle damage markers, including CK and serum myoglobin levels, at all follow-up visits (Tables 2 and 3).

Transcriptome profiling of skeletal muscle following oral PVS supplementation

To determine the changes in the transcriptomes of human skeletal muscle in response to oral PVS supplementation, muscle samples were collected during each visit. RNA extraction, target labeling, and GeneChip data analysis were performed using Affymetrix® Human Transcriptome Array 2.0 (HTA 2.0) as described previously.^{18,19} The HTA 2.0 contains >6.0 million probes covering coding transcripts and exon-exon splice junctions and noncoding transcripts.²³ A total of 175 annotated probe sets were differentially ($P < .01$) regulated following 8-week supplementation compared with baseline visits (Fig. 1A). Top 20 candidates based on fold change (compared with baseline) that was altered (up or downregulated) in the supplementation group have been provided in Tables 4 and 5. Pathway analysis revealed an ECM-related cluster of probe sets that was significantly upregulated in the 8-week supplementation group compared with corresponding baseline visit (Fig 1B). This probe set included ECM genes: tenascin XB (*TNXB*),

TABLE 4. GENES UPREGULATED IN SKELETAL MUSCLES FOLLOWING ORAL PVS SUPPLEMENTATION

Transcript cluster ID	Gene symbol	Gene description	Fold change	P
18723098	<i>COL3A1 MIR3606</i>	Collagen, type III, alpha1 microRNA3606	5.18	.0087
18811819	<i>COL1A2</i>	Collagen, type I, alpha2	5.13	.0077
18942601	<i>MMP2</i>	Matrixmetalloproteinase2 (gelatinaseA, 72 kDa gelatinase, 72 kDa type IV collagenase)	3.73	.0100
18737057	<i>FNI</i>	Fibronectin1	3.65	.0085
18798512	<i>FNDC1</i>	Fibronectin type III domain containing 1	3.30	.0006
18817641	<i>SFRP4</i>	Secreted frizzled-related protein 4	3.14	.0031
18847049	<i>ASP</i>	Asporin	2.32	.0002
18830114	<i>COL14A1</i>	Collagen, type XIV, alpha1	2.08	.0061
18864477	<i>PLXDC2</i>	Plexin domain containing 2	1.83	.0020
18907603	<i>LUM</i>	Lumican	1.77	.0017
18693079	<i>HMCN1</i>	Hemicentin1	1.71	.0038
18915509	<i>LHFP</i>	Lipoma HMGIC fusion partner	1.69	.0015
18960437	<i>MFAP4</i>	Microfibrillar-associated protein4	1.67	.0075
18717539	<i>ANTXR1</i>	Anthrax toxin receptor 1	1.61	.0041
18936069	<i>ITGA11</i>	Integrin, alpha11	1.56	.0058
18828471	<i>SULF1</i>	Sulfatase1	1.36	.0038
18786580	<i>EDIL3</i>	EGF-like repeats and discoid in I-like domains 3	1.33	.0067
18716082	<i>LTBP1</i>	Latent transforming growth factor beta-binding protein 1	1.33	.0005
18956362	<i>MRC2</i>	Mannose receptor, Ctype2	1.32	.0035
19022949	<i>RNU5E-1</i>	RNA, U5E small nuclear 1	1.31	.0053

Data presented indicate fold changes in expression of PVS-sensitive genes following 8 weeks of oral PVS supplementation compared with corresponding baseline visits. Transcripts cluster ID, Affymetrix probe identifications. Data correspond to Figure 1A, cluster a ($P < .05$; FDR $< 5\%$).

decorin (*DCN*), collagen (*COL*) (type I, III, V, VI, XIV), fibrillin 1 (*FBNI*), elastin (*ELN*), myoferlin (*MYOF*), and fibronectin 1 (*FNI*) (Table 6). Among these upregulated genes, *TNXB* was increased by ~1.7-fold and *DCN* was increased by 2.23- and 1.09-fold, respectively (Table 6). *COL1A1*, *COL1A2*, and *COL3A1* were increased by 4.61-, 5.13-, and 5.18-fold, respectively, and *ELN*, *FBNI*, and *FNI*

were increased by 1.13-, 3.05-, and 3.65-fold, respectively (Table 6).

Validation of GeneChip data using RT-PCR

To validate the genes identified using microarray analysis, RT-PCR was performed. Since the above-mentioned

TABLE 5. GENES DOWNREGULATED IN SKELETAL MUSCLES FOLLOWING ORAL PVS SUPPLEMENTATION

Transcript cluster ID	Gene symbol	Gene description	Fold change	P
19534964	<i>SNAR-G2</i>	Small ILF3/NF90-associated RNA G2	-1.27	.0023
19140074	<i>MIR4792</i>	MicroRNA 4792	-1.24	.0065
18729405	<i>SIX2</i>	SIX homeobox 2	-1.23	.0057
18928262	<i>KLF13</i>	Kruppel-like factor 13	-1.22	.0035
19195587	<i>MIR4635</i>	MicroRNA 4635	-1.21	.0008
18820492	<i>VEGF</i>	VEGF nerve growth factor inducible	-1.21	.0095
19160216	<i>DRD5</i>	dopamine receptor D5	-1.21	.0003
18949268	<i>GCSH</i>	Glycine cleavage system protein H (amino methyl carrier)	-1.20	.0076
19113156	<i>MIR3132</i>	MicroRNA 3132	-1.20	.0006
18939902	<i>CLDN9</i>	Claudin 9	-1.20	.0035
18999582	<i>IGLV2-33</i>	Immunoglobulin lambda variable 2-33 (nonfunctional)	-1.19	.0054
18885915	<i>MIR210HG</i>	MIR210 host gene (nonprotein coding)	-1.19	.0000
18739218	<i>IGKV1-37</i>	Immunoglobulin kappa variable 1-37 (nonfunctional)	-1.18	.0024
19043031	<i>LOC100287934</i>	LOC100287934	-1.17	.0072
19499270	<i>MIR4734</i>	MicroRNA 4734	-1.17	.0037
19222951	<i>LINC00602</i>	Long intergenic nonprotein coding RNA 602	-1.16	.0008
18979687	<i>TICAM1</i>	Toll-like receptor adaptor molecule 1	-1.16	.0031
19570418	<i>RNS5497</i>	RNA, 5S ribosomal 497	-1.15	.0027
18800247	<i>IER3</i>	Immediate early response 3	-1.15	.0021
19524368	<i>SPACA4</i>	Sperm acrosome associated 4	-1.14	.0034
18889853	<i>YIF1A</i>	Yip1 interacting factor homolog A (<i>Saccharomyces cerevisiae</i>)	-1.14	.0003

Data presented indicate fold change in expression of PVS-sensitive genes following 8 weeks of oral PVS supplementation compared with corresponding baseline visits. Transcripts cluster ID, Affymetrix probe identifications. Data correspond to Figure 1A, cluster b ($P < .05$; FDR $< 5\%$).

TABLE 6. LIST OF ECM-RELATED UPREGULATED GENES IN SKELETAL MUSCLES FOLLOWING ORAL PVS SUPPLEMENTATION

Transcript cluster ID	Gene symbol	Gene description	Fold change	P
19015271	TNXB	Tenascin XB	1.78	.0311
19011964	TNXB	Tenascin XB	1.76	.0467
18807632	TNXB	Tenascin XB	1.75	.0425
19018936	TNXB	Tenascin XB	1.74	.0427
19012977	TNXB	Tenascin XB	1.71	.0430
18907606	DCN	Decorin	2.23	.0186
18907622	DCN	Decorin	1.09	.0338
18874445	MYOF	Myoferlin	1.11	.0207
18963453	COL1A1	Collagen, type I, alpha1	4.61	.0149
18811819	COL1A2	Collagen, type I, alpha2	5.13	.0076
18723098	COL3A1/ MIR3606	Collagen, type III, alpha1/microRNA3606	5.18	.0086
18735673	COL5A2	Collagen, type V, alpha2	1.62	.0324
18738278	COL6A3	Collagen, type VI, alpha3	2.96	.0122
18830114	COL14A1	Collagen, type XIV, alpha1	2.07	.0061
18810809	ELN	Elastin	1.13	.0187
18934479	FBN1	Fibrillin1	3.05	.0254
18737057	FNI	Fibronectin1	3.65	.0084

Data presented indicate fold change in expression of ECM-related genes following 8 weeks of oral PVS supplementation compared with corresponding baseline visits. Data correspond to Figure 1B ($P < .05$; FDR $< 5\%$). ECM, extracellular matrix.

ECM-related genes were upregulated, those genes were re-examined. Consistent with microarray results, significant upregulation of collagen and other ECM-associated genes was noted in the muscle samples following 8 weeks of oral PVS supplementation compared with the baseline visit (Fig. 2 and Table 7). Interestingly, additional 4 weeks of oral supplementation with exercise further induced the expression of the microarray-identified genes (Fig. 2 and Table 7).

DISCUSSION

Beneficial effects of Shilajit in skeletal muscle adaptation have been highlighted for centuries in ancient Ayurveda medicine.^{4,5} The underlying mechanisms of such adaptation have not been elucidated. The current study, for the first time, presents a mechanism of action of Shilajit in improving skeletal muscle adaptation in overweight/obese subjects exercising 30 min a day 3 days a week for 4 weeks. ECM plays an essential role in the development, maintenance, and regeneration of skeletal muscles.^{24,25} Chronic loading of muscles such as physical training leads to augmented synthesis and turnover of ECM.²⁶ Oral supplementation of PVS markedly enhanced ECM-related gene expression in overweight/class I obese human subjects. PVS is one of the very few nutritional supplements that induce skeletal muscle adaptation through upregulation of ECM genes.

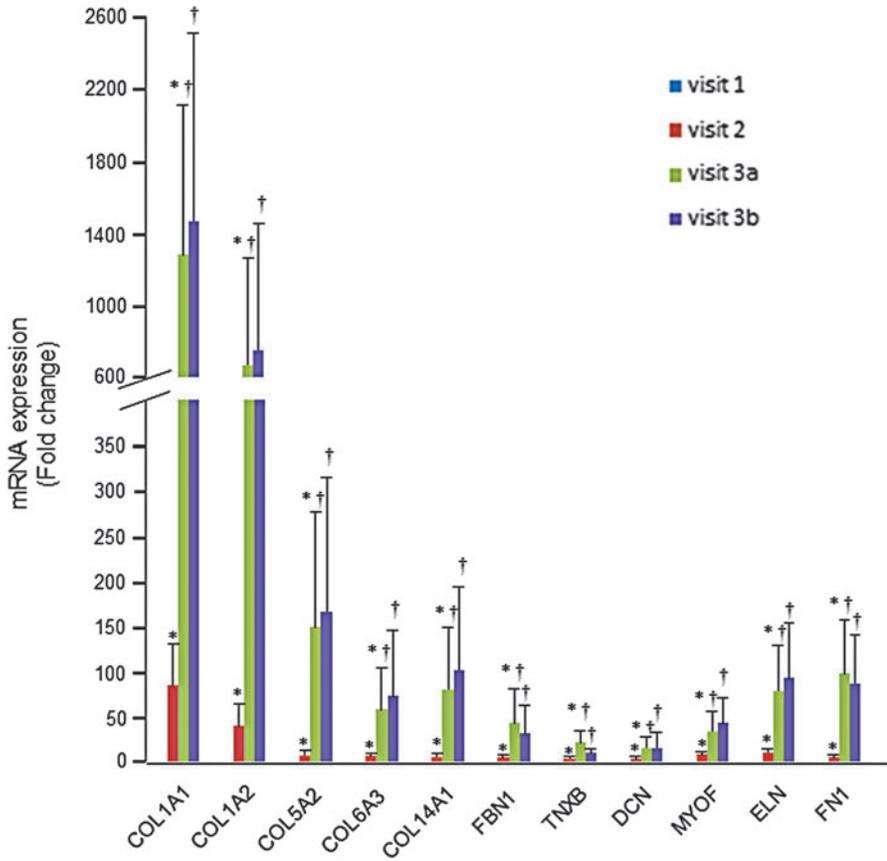


FIG. 2. RT-PCR validation of ECM-related genes derived from microarray analysis following oral PVS supplementation. Expression levels of selected collagen genes identified using GeneChip® analyses were independently verified using real-time quantitative (Q) PCR. The effects of oral PVS supplementation (250 mg/b.i.d.) were measured during the course of all visits; V1, baseline; V2, after 8 weeks of oral supplementation; V3a, additional (following 8 weeks of initial supplementation) 4 weeks of oral supplementation and exercise, sample collection before the final stint of exercise; and V3b, same as study visit 3, sample collected 30 min post-final bout of exercise. Data are mean ± SEM (n = 16); *P < .05 compared with the baseline visit and †P < .05 compared with 8 weeks. No significant changes were observed between pre and post 30-min final exercise on week 12. PCR, polymerase chain reaction.

TABLE 7. MULTIVARIATE TEST OF ALL ECM-RELATED GENES (JOINTLY)

Comparison	Contrast	Standard Error	Z	P
8 weeks versus baseline	-3.68	1.22	-3.02	.003
9-12 weeks (pre-final exercise) versus baseline	-6.47	1.22	-5.30	<.001
9-12 weeks (pre-final exercise) versus 8 weeks	-2.79	1.22	-2.29	.022
9-12 weeks (post-final exercise) versus 8 weeks	-2.56	1.22	-2.10	.035
9-12 weeks (post-final exercise) versus 9-12 weeks (pre-final exercise)	0.22	1.22	0.18	.855

During obesity, there is an increase in circulating lipids (free fatty acids, triglycerides) that accumulate in muscle as triacylglycerol as well as fatty acid metabolites such as ceramide, diacylglycerol, and long-chain acyl CoA.²⁷ No alterations in blood lipid profiles of subjects after supplementation suggest that PVS was well tolerated in this class of human subjects. Phosphocreatine (PCr) is one major source of ATP replenishment in tissues with rapidly shifting energy demand.²⁸ The CK reaction mediates this supply, in which creatine and adenosine diphosphate (ADP) are reversibly phosphorylated to PCr and ATP, respectively.²⁸ Functioning as a spatial and temporal buffer of ATP levels, the PCr-CK system requires a high level of total cellular creatine in mammal skeletal muscle.²⁹ So, reduction in CK may disturb ATP formation within skeletal muscle. High intracellular creatine concentrations are achieved by a combination of exogenous dietary intake and endogenous production, followed by cellular uptake of creatine from blood vessels.²⁹ The unaffected levels of serum CK in our study provided evidence for skeletal muscle integrity following oral supplementation. The high amounts of myoglobin in the skeletal and cardiac muscles enable storage and diffusion of oxygen in these tissues.³⁰ The unchanged levels of serum myoglobin and blood glucose on all visits confirmed that PVS was well tolerated and maintained physiological body glucose metabolism, homeostasis, and muscle integrity in the skeletal muscle of overweight/class I obese human subjects.

Skeletal muscle is highly plastic and well known to undergo significant adaptive modifications in response to both endurance and resistance exercise.³¹⁻³⁴ Increasing evidences now indicate that in response to nutrition, skeletal muscle undergoes adaptive changes through regulatory processes driven by changes in gene expression and cell signaling.^{35,36} ECM of skeletal muscle mainly comprises glycoproteins, collagen, and proteoglycans and plays a major role in mechanotransduction, that is, conducting force laterally between fibers and tendons.³⁷⁻³⁹ In our study, both microarray and RT-PCR results revealed elevated mRNA expression of collagen (type I, III, V, VI, and XIV) in response to oral PVS supplementation. The major structural protein in skeletal muscle ECM, collagen, comprises 1% to 2% of the muscle tissue and represents 6% of the weight of tendinous muscles.

The skin comprises mainly collagen type I, which constitutes about 70% of collagen, with type III being 10% and trace amounts of collagen types IV, V, VI, and VII. With strenuous exercise, a rapid increase in the synthesis of collagen in tendons and muscles has been noted in mice and humans.^{26,40} Increase in the expression of *COL1A2*, *COL3A1*, and *COL5A1* genes enhances cell proliferation and active remodeling of ECM in tissue repair.⁴¹ Exercise-induced ECM synthesis leads to protein degradation through increased matrix metalloproteinase (MMP) activity.²⁶ Microarray data show an increase in *MMP-2* gene expression in muscles following oral supplementation, suggesting that the effects of PVS supplementation on skeletal muscle adaptation are comparable with exercise by mediating specific synthesis and degradation of ECM. Growth factors such as *TGF- β* and *IGF-1* are involved in regulation of ECM synthesis in connective tissue.²⁶ The concentration of these growth factors has been shown to increase following exercise. Interestingly, increased expression of *IGF1R* was noted through microarray analysis. It is plausible that PVS induces ECM gene expression through comparable mechanisms. Further studies are required to determine exact mechanisms of PVS-induced ECM gene expression changes.

Although collagen provides the main structure, other ECM components also play an important role in skeletal muscle adaptation. In our study, both microarray and RT-PCR results revealed elevated mRNA expression of other ECM components including decorin, fibronectin, fibrillin, tenascin XB, myoferlin, and elastin, in response to oral PVS supplementation. Decorin is a small leucine-rich proteoglycan and contributes both to the formation and stabilization of collagen fibers in the perimysium that support muscle fibers assembled with myogenesis.⁴² Fibronectin plays a major role in synthesizing provisional granulation tissue during the early phases of wound repair.⁴¹ Fibrillin, a type of microfibril, is also one of the key structural elements in the ECM of skeletal muscle. Being widely distributed in connective tissues, fibrillins are arranged in tissue-specific architectures.^{43,44} The other ECM component, tenascin XB, determines the mechanical properties of collagen.⁴⁵ During the development of muscles, especially during myoblast fusion, myoferlin is highly expressed⁴⁶ and regulates the reutilizing of vascular endothelial growth factor receptor-2.⁴⁷ The levels of myoferlin are generally less in adult skeletal muscle and almost lacking in healthy myofibers. An increase in the myoferlin level leads to a buildup of mononuclear myoferlin-positive myoblasts that play a key role in the repair of damaged myofibers, suggesting the importance of this gene in muscle repair and regeneration.⁴⁶

CONCLUSIONS

The current study reports for the first time that oral supplementation of a natural product to overweight/class I obese human subjects resulted in skeletal muscle adaptation through upregulation of ECM-related genes that control muscle mechanotransduction properties, elasticity, repair, and regeneration.

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