US 20160220609A1

(19) **United States**

(12) **Patent Application Publication**

Sen

(10) **Pub. No.: US 2016/0220609 A1**

(43) **Pub. Date: Aug. 4, 2016**

(54) **REGULATION OF STEROIDOGENIC ACTIVITY BY USING PURIFIED SHILAJIT**

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(21) Appl. No.: **14/917,873**

(22) PCT Filed: **Sep. 9, 2014**

(86) PCT No.: **PCT/US2014/054705**

§ 371 (c)(1),
(2) Date: **Mar. 9, 2016**

Related U.S. Application Data

(60) Provisional application No. 61/875,513, filed on Sep. 9, 2013.

Publication Classification

(51) **Int. Cl.**
A61K 35/04 (2006.01)
A61K 35/66 (2006.01)

(52) **U.S. Cl.**
CPC ***A61K 35/04*** (2013.01); ***A61K 35/66*** (2013.01)

(57) **ABSTRACT**

A method of using Purified Shilajit to promote steroidogenic activity in a mammal is provided.

REGULATION OF STEROIDOGENIC ACTIVITY BY USING PURIFIED SHILAJIT

TECHNICAL FIELD

[0001] The present invention relates to promoting steroidogenic activity in the body of a mammal, including human, through the use of Shilajit.

[0002] BACKGROUND

[0003] Shilajit is composed of rock humus, rock minerals and organic substances that have been compressed by layers of rock mixed with marine organisms and microbial metabolites. It oozes out of the rocks in the Himalayas at higher altitudes ranging from 1000-5000 meters as black mass and is regarded as a maharasa (super-vitalizer) in Ayurveda, the traditional Indian system of medicine, dating back to 3500 B.C. Shilajit contains fulvic acids as the main components along with dibenzo-a-pyrones ("DBPs") and dibenzo-a-pyrone chromoproteins.

[0004] Fulvic acid complex, derived from shilajit, is an assembly of naturally occurring low and medium molecular weight compounds comprising oxygenated dibenzo-alpha-pyrones (DBPs), both in reduced as well as in oxidized form, as the core nucleus, and acylated DBPs and lipids as partial structural units, along with fulvic acids ("FAs"). Fulvic acid complex material derived from alluvial sources lack DBPs; instead, the core nucleus of alluvial fulvic acid is comprised of benzoic acid.

[0005] Thus, the active constituents of shilajit contain dibenzo-alpha-pyrones and related metabolites, small peptides (constituting non-protein amino acids), some lipids, and carrier molecules (fulvic acids). See, Ghosal, S., et al., "Shilajit Part 1—Chemical constituents," *J. Pharm. Sci.* (1976) 65:772-3; Ghosal, S., et al., "Shilajit Part 7—Chemistry of Shilajit, an immunomodulatory ayurvedic rasayana," *Pure Appl. Chem.* (IUPAC) (1990) 62:1285-8; Ghosal, S., et al., "The core structure of Shilajit humus," *Soil Biol. Biochem.* (1992) 23:673-80; and U.S. Pat. Nos. 6,440,436 and 6,869,612 (and references cited therein); all hereby incorporated by reference herein.

[0006] Shilajit finds extensive use in Ayurveda, for diverse clinical conditions. For centuries people living in the isolated villages in Himalaya and adjoining regions have used Shilajit alone, or in combination with, other plant remedies to prevent and combat problems with diabetes (Tiwari, V. P., et al., "An interpretation of Ayurvedica findings on Shilajit," *J. Res. Indigenous Med.* (1973) 8:57). Moreover being an antioxidant it will prevent damage to the pancreatic islet cell induced by the cytotoxic oxygen radicals (Bhattacharya S. K., "Shilajit attenuates streptozotocin induced diabetes mellitus and decrease in pancreatic islet superoxide dismutase activity in rats," *Phytother. Res.* (1995) 9:41-4; Bhattacharya S. K., "Effects of Shilajit on biogenic free radicals," *Phytother. Res.* (1995) 9:56-9; and Ghosal, S., et al., "Interaction of Shilajit with biogenic free radicals," *Indian J. Chem.* (1995) 34B: 596-602). It has been proposed that the derangement of glucose, fat and protein metabolism during diabetes, results into the development of hyperlipidemia. In one study, Shilajit produced significant beneficial effects in lipid profile in rats (Trivedi N. A., et al., "Effect of Shilajit on blood glucose and lipid profile in alloxan-induced diabetic rats," Indian J. Pharmacol. (2004) 36(6):373-376).

[0007] As discussed, shilajit has been used to treat various ailments. It is also recommended as a performance enhancer. Fulvic acids (FAs) are reported to elicit many important roles in biological systems of plants, in animals as well as humans, including: (a) improvement of bioavailability of minerals and nutrients, (b) serve as electrolytes, (c) detoxification of toxic substances including heavy metals, (d) perform as antioxidants, and (e) improvement of immune function.

[0008] Furthermore, dibenzo-a-pyrones have been hypothesized to participate in the electron transport inside the mitochondria, thus facilitating production of more ATP, leading to increased energy. Thus, shilajit is found to increase energy, among other beneficial qualities.

[0009] In view of the above, it would be desirable to provide a method of using shilajit for improvement of mitochondrial function thus increasing energy in a human or animal. If a way could be found to stimulate steroidogenic gene expression related to skeletal muscle activity to provide increased energy using Shilajit, this would provide a valuable contribution to the medical and nutritional arts.

SUMMARY

[0010] An objective of the present invention is to develop a method of using Shilajit for promoting steroidogenic activity in the body of a mammal, for example, a human.

[0011] A method for promoting steroidogenic activity in a mammal is provided, comprising administering to the mammal in need of such treatment an effective amount of a purified Shilajit, wherein energy levels in the mammal are increased.

DETAILED DESCRIPTION

[0012] In one embodiment a gene expression study was conducted on the skeletal muscle of mice with Shilajit, 3,8-dihydroxy-dibenzo-a-pyrone (3,8-$(OH)_2$-DBP), and placebo to determine the effect of these compounds on expression of genes related to skeletal muscle activity.

[0013] In another embodiment, a human clinical study was conducted with supplementation of Purified Shilajit for 8 weeks and skeletal muscle tissue was analyzed for gene expression.

[0014] It is contemplated that the compositions used herein may be administered advantageously in a mammal for inducing or promoting steroidogenic activity. As used herein, a mammal may include, but is not limited to, a human, a dog, a horse, or a cat.

[0015] Materials: Purified Shilajit (PrimaVie®, Natreon, Inc., New Brunswick, N.J.) is a standardized dietary supplement ingredient extracted and processed from Shilajit bearing rocks, containing not less than about 50% by weight fulvic acids (FAs), at least about 10% by weight dibenzo-a-pyrone chromoproteins, and at least 0.3%, or more, by weight total dibenzo-a-pyrones (DBPs).

[0016] 3,8-$(OH)_2$-DBP (99.0% pure, Natreon, Inc., New Brunswick, N.J.).

[0017] Procedure for Studies in Mice Using Shilajit and DBPs:

[0018] Three groups of adult mice (n=8) were intragastrically supplemented with purified Shilajit (PS), 3,8-$(OH)_2$-DBP, or placebo for 12 weeks. At the end of week 12, skeletal muscle tissue was harvested for gene profiling. Some tissue was stored for histology and HPLC analysis.

[0019] The control group of mice received DMSO in corn oil while the PS group received 100 mg of purified Shilajit/kg

body weight of mice, dissolved in water and the DBP group received 10 mg of 3,8-$(OH)_2$-DBP/kg body weight of mice, dissolved in DMSO/corn oil.

[0020] At week 12, the following tissues were collected from mice: heart, lung, liver, brain, muscles, adipose tissue, skeletal muscle (vastus lateralis) and whole blood.

[0021] Procedure for Human Clinical Study:

[0022] 20 healthy volunteers were recruited following proper procedures for clinical studies. The baseline readings were taken and supplementation with Purified Shilajit 250 mg twice/day dosing was done for 8 weeks. Skeletal muscle biopsy was done and the tissue collected was subjected to gene chip analysis as described below.

[0023] Gene Expression Profiling using GeneChip® Assay

[0024] Affymetrix GeneChip® technology (Affymetrix, Santa Clara, Calif.) was used for transcriptome profiling of skeletal muscle tissue. Gene chip assays were performed in accordance with the following references: Roy, S., Biswas, S., Khanna, S., Gordillo, G., Bergdall, V., Green, J., Marsh, C. B., Gould, L. J., Sen, C. K., "Characterization of a preclinical model of chronic ischemic wound," Physiol. Genomics (2009) May 13; 37(3):211-24; Roy, S., Khanna, S., Rink, C., Biswas, S., Sen, C. K., "Characterization of the acute temporal changes in excisional murine cutaneous wound inflammation by screening of the wound-edge transcriptome," Physiol. Genomics (2008) Jul. 15; 34(2):162-84; and Roy, S., Patel D, Khanna, S., Gordillo, G. M., Biswas, S., Friedman, A., Sen, C. K., "Transcriptome-wide analysis of blood vessels laser captured from human skin and chronic wound-edge tissue," Proc. Natl. Acad. Sci. USA (2007) Sep. 4; 104(36):14472-7; herein incorporated by reference.

[0025] Results:

[0026] The following genes for steroid biosynthesis were up regulated or induced in mice by Shilajit:

[0027] (1) Hsd3b5: hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5.

[0028] (2) Stard3: START domain containing 3. Start domain-containing protein 3; STARD3, a.k.a. metastatic lymph node 64: MLN64. Expression of MLN64 leads to increased pregnenolone secretion and that steroidogenic activity resides in the C terminus of the protein. Pregnenolone, also known as 3α,5β-tetrahydroprogesterone (3α, 5β-THP), is an endogenous steroid hormone involved in the steroidogenesis of progestogens, mineralocorticoids, glucocorticoids, androgens, and estrogens, as well as the neuroactive steroids.

[0029] (3) Star: steroidogenic acute regulatory protein. Studies of Star in MA10 cells in the absence of hormone stimulation was sufficient to induce steroid production. This study concluded that Star is required for hormone-induced steroidogenesis.

[0030] (4) HSD3B1: 3-beta-hydroxysteroid dehydrogenase 1. 3-Beta-hydroxysteroid dehydrogenase catalyzes the oxidation and isomerization of delta-5-3-beta-hydroxysteroid precursors into delta-4-ketosteroids, thus leading to the formation of all classes of steroid hormones.

[0031] The steroidogenic genes may be up-regulated by Shilajit in accordance with an embodiment of the present invention. Other steroidogenic genes that may be upregulated include, but are not limited to: androgen binding protein alpha (Abpa), and oxysterol binding protein 2 (Osbp2).

[0032] 3,8-$(OH)_2$-DBP did not show significant effect on steroidogenic activity in mice.

[0033] Table 1 shows fold change results for several representative steroidogenic genes, in accordance with a hierarchical gene cluster array showing genes up-regulated in mice treated with Purified Shilajit. In particular, these genes are demonstrating up-regulation or induction in muscle tissue with Purified Shilajit.

TABLE 1

Steroidogenic Genes Up Regulated in Mouse Skeletal Muscle by Shilajit

Gene Symbol	Gene Title	mean	p value
Hsd3b5	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5	1.10	0.020
Stard3	START domain containing 3	1.22	0.024
Star	steroidogenic acute regulatory protein	1.22	0.010
Abpa	androgen binding protein alpha	1.15	0.002
Osbp2	oxysterol binding protein 2	1.11	0.026

[0034] Table 2 shows fold change for several steroidogenic genes in the human clinical study. These results are based on gene chip analysis of skeletal muscle samples from three subjects out of a total of 20 subjects. In particular, these genes are demonstrating up-regulation or induction in muscle tissue with Purified Shilajit. Gene chip analysis of the samples from the remaining subjects is pending and the statistical significance of these results is expected to improve after the results when all 20 subjects are statistically analyzed.

[0035] Additional animal and/or human studies are expected to further demonstrate the steroidogenic activity of Shilajit.

TABLE 2

Steroidogenic Genes Up Regulated in Human Skeletal Muscle by Shilajit

Gene Symbol	Gene Title	Fold Change
HSD17B6	hydroxysteroid(17-beta)dehydrogenase6homolog (mouse)	1.0015
SRD5A1	steroid-5-alpha-reductase,alphapolypeptide1(3-oxo-5alpha-steroiddelta4-dehydrogenasealpha1)	1.0024
HSD17B3	hydroxysteroid(17-beta)dehydrogenase3	1.0010
HSD17B1	hydroxysteroid(17-beta)dehydrogenase1	1.0009
HSD17B8	hydroxysteroid(17-beta)dehydrogenase8	1.0032
HSD17B8	hydroxysteroid(17-beta)dehydrogenase8	1.0024
HSD17B8	hydroxysteroid(17-beta)dehydrogenase8	1.0022
HSD17B8	hydroxysteroid(17-beta)dehydrogenase8	1.0015
HSD17B8	hydroxysteroid(17-beta)dehydrogenase8	1.0027
HSD17B8	hydroxysteroid(17-beta)dehydrogenase8	1.0024
HSDL2	hydroxysteroiddehydrogenaselike2	1.0062
STAR	steroidogenicacuteregulatoryprotein	1.0003
HSD17B10	hydroxysteroid(17-beta)dehydrogenase10	1.0035
SRD5A2	steroid-5-alpha-reductase,alphapolypeptide2(3-oxo-5alpha-steroiddelta4-dehydrogenasealpha2)	1.0008
HSD3B2	hydroxy-delta-5-steroiddehydrogenase,3beta-andsteroiddelta-isomerase2	1.0006
HSD11B2	hydroxysteroid(11-beta)dehydrogenase2	1.0087
SRA1	steroidreceptorRNAactivator1	1.0006
HSDL1	hydroxysteroiddehydrogenaselike1	1.0093

[0036] The product(s) of the present invention may be formulated into nutraceutical or pharmaceutical dosage forms comprising of tablets, capsules, powders, liquids, chews, gummies, transdermals, injectables, etc. using standard excipients and formulation techniques in the industry. The

product of the subject invention may be administered to the mammal orally in solid dosage form or by parenteral or transdermal administration.

[0037] While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been put forth for the purpose of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein can be varied considerably without departing from the basic principles of the invention.

[0038] All references cited herein are incorporated by reference in their entirety. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

1. A method for promoting steroidogenic activity in a mammal, comprising administering to the mammal in need of such treatment an effective amount of a purified Shilajit, wherein energy levels in the mammal are increased.

2. The method of claim **1**, wherein the compound is administered orally, intramuscularly, parenterally, or transdermally.

3. The method of claim **1**, wherein the mammal is a human, a dog, a horse, or a cat.

4. The method of claim **1**, wherein the purified Shilajit is present in a daily dosage of from about 1.0 mg/kg body weight of the mammal to about 20 mg/kg body weight of the mammal.

5. The method of claim **1**, wherein energy levels are determined by muscular activity.

6. The method of claim **5**, wherein the muscular activity is characterized by increased induction of one or more genes selected from the group consisting of: hsd3b5, stard3, star, abpa, osbp2, hsdl7b6, srd5al, hsdl7b3, hsdl7b1, hsdl7b8, hsd12, hsdl7b10, srd5a2, hsd3b2, hsdl1b2, sra1, and hsdl1.

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