Clinical evaluation of spermatogenic activity of processed Shilajit in oligospermia

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Clinical evaluation of purified Shilajit on testosterone levels in healthy volunteers

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Summary

Purified Shilajit, an Ayurvedic *rasayana*, was evaluated in healthy volunteers of age between 45 and 55 years for its effect on male androgenic hormone *viz.* testosterone in a randomised, double-blind, placebo-controlled clinical study at a dose of 250 mg twice a day. Treatment with Shilajit for consecutive 90 days revealed that it has significantly ($P < 0.05$) increased total testosterone, free testosterone and dehydroepiandrosterone (DHEAS) compared with placebo. Gonadotropic hormones (LH and FSH) levels were well maintained.

Introduction

Purified Shilajit (PS) is used in Ayurveda, indigenous system of Indian medicine, as a remedy for several diseases, particularly chronic diseases. Shilajit is a pale-brown to blackish-brown exudate that oozes from sedimentary rocks worldwide, largely in the Himalayas. Common people describe it from their knowledge as pahar-ki-pasina (sweat of mountains), paharki-khoon (mountain blood), shilaras (rock juice), asphalt, bitumen, etc. Shilajit is said to carry the healing power of these great mountains (David & Vasant, 2001). It is an important drug of the ancient Ayurvedic materia medica and it is to this day used extensively by Ayurvedic physicians for a variety of diseases. Early Ayurvedic writings from the Charaka Samhita (Sharma, 1998) describe Shilajit as a cure for all diseases as well as a *Rasayana* (rejuvenator) that promises to increase longevity. It 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 (Ghosal, 1994).

Traditional uses of Shilajit primarily focus not only on diabetes and diseases of the urinary tract, but also on oedema, tumours, muscle wasting, epilepsy and even insanity. Modern indications extend to all systems of the human body with a significant number of additions in the reproductive and nervous system. Clinical research confirms many of the properties for which Shilajit has been used (Talbert, 2004). In Ayurveda, Shilajit is employed for the management of male reproductive disorders, and in particular, under the parlance of *Vrisya* (an aphrodisiac with special reference to spermatogenesis) (Sharma, 1998).

Several toxicological studies, both acute and subchronic, have already been performed by many scientists with Shilajit throughout the world. Oral LD50 was found to be >2000 mg kg^{-1} (Acharya *et al.*, 1988; Ghosal *et al.*, 1989), and Shilajit was proved to be safe at doses of 0.2–1.0 g per kg body weight when used chronically (Kelginbaev *et al.*, 1973; Anisimov & Shakirzyanova, 1982; Fortan & Acharya, 1984; Al-Hamaidi & Umar, 2003). Clinical evaluation of spermatogenic activity of processed shilajit in oligospermia (Biswas *et al.*, 2009) revealed that there was no alteration on objective features related to any systemic toxicities such as serum urea, uric acid, serum bilirubin, total protein, serum globulin, SGPT, SGOT and alkaline phosphatase (Biswas *et al.*, 2009). Besides, it was also observed that there was a significant ($P < 0.001$) improvement in spermia (+37.6%), total sperm count (+61.4%), motility (12.4–17.4% after different time intervals), normal sperm count (+18.9%) and total testosterone (+23.5%) with concomitant decrease in pus and epithelial cell count compared with baseline value in 28 patients of oligospermia after 90 days of treatment with PS at a dose of 100 mg twice daily. An unpublished human safety study of purified Shilajit from Natreon, Inc., New Brunswick, NJ, USA by the present

Andrologia 2015, **xx**, 1–6

authors has demonstrated the safety of this product at 250 mg twice a day dosing (Jana, Utpalendu, Biswas, Tuhin; J.B.Roy Ayurvedic Medical College and Hospital, Kolkata, India, Project No.: JBR/Res/01/2007), and an unpublished animal study in rats with 100 mg/kg b.w. (equivalent to human dose of 850 mg) by Natreon showed a significant increase in testosterone levels. Thus, a dose of 250 mg twice daily was selected for this study.

Materials and methods

Preparation and analysis of the drug

Purified Shilajit (PrimaVie™, Kolkata, West Bengal, India), a patented (US 6,440,436, 6,969,612, 6,558,712, EP 1 387 614) standardised extract of native Shilajit from Natreon, Inc., was used for the study. Shilajit was standardised to contain not less than 60% w/w of total bioactives, which include not less than 50% w/w of fulvic acids (FAs), not less than 0.3% w/w of dibenzo α-pyrones (DBPs) and not less than 10% w/w of dibenzo-α-pyrone chromoproteins (DCPs), as quantified by HPLC using external standards (isolated from Shilajit extract by low pressure (Lobar) chromatography). An HPLC chromatogram of this product is shown in Fig. 1.

Selection of healthy volunteers

The clinical trial was conducted between December 2012 and March 2014 at J. B. Roy State Ayurvedic Medical College and Hospital, Kolkata, India, after obtaining necessary permission from Institutional Ethics Committee (IEC) of J. B. Roy State Ayurvedic Medical College and Hospital, Kolkata, India. A schematic of the study design is shown in Fig. 2. Healthy volunteers aged between 45 and 55 years, irrelevant of religion, income status and occupation, were selected for the present purpose, and the distribution of patients was done by the method of double-blind randomised techniques. Initially, 145 volunteers were selected on the basis of primary assessment eligibility and 49 among them were excluded for various reasons (Fig. 2). A total of 96 volunteers were enrolled in the present trial and randomly divided into two equal groups as PS treated and placebo treated, each with 48 subjects. In the course of the study, 21 subjects discontinued for various reasons and 38 subjects in PS-treated group and 37 subjects in the placebo group completed the study (Fig. 2). Mean age of the volunteers was 49.44 years in the test drug group and 48.89 years in the placebo group, and thus, there is no bias due to the difference in mean ages. Volunteers were included in the study after taking their consent in trilingual (English, Bengali and Hindi) using prescribed format of WHO-Helsinki rules. History of volunteers was taken according to the standard protocol mentioning name, age, sex, religion, address, occupation, income status, history of past illness, family history, personal history, marital history, general examination, systemic examination, laboratory investigation, etc.

Dosage regimen

Both the groups received respective drugs in the dose of 250 mg/capsule orally, twice daily after major meals, for a

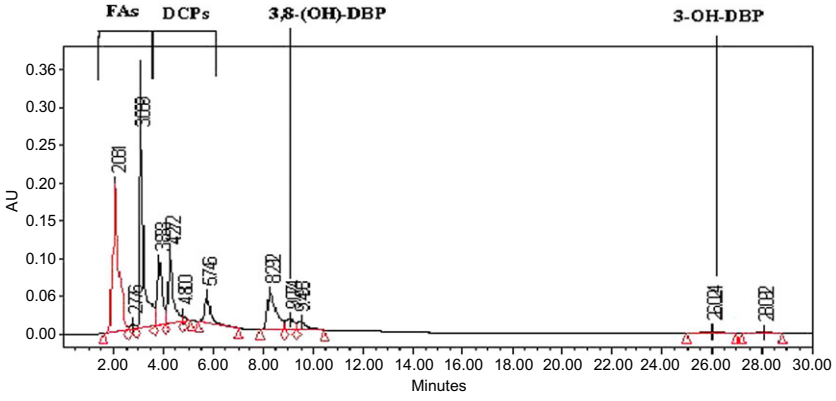

Fig. 1 HPLC chromatogram of Shilajit (PrimaVie™) by RP-C 18 column. Fulvic acids (FAs) t_R: 2.0–3.0 min; Dibenzochromo proteins (DCPs) t_R: 3.0–5.8 min; 3,8 -$(OH)_2$ –Dibenzo-α-pyrone t_R: 9.07 min; 3-OH- Dibenzo-α-pyrone t_R: 26.02 min. HPLC analysis was performed under ambient conditions, with Waters HPLC equipment comprising Waters 515 pumps, Waters photodiode array detector (PDA) model 2996, Waters pump controller module and EMPOWER software (version 1) Natreon Inc., New Brunswick, New Jersey, USA. Samples (20 µl) were injected by means of a Rheodyne injector fitted with a 20-µl loop. Compounds were separated on a C18 reverse-phase (Lichrocart® column (250 mm × 4 mm, i.d., 5-µm particle; column no 331303, Darmstadt, Germany) using the mobile phase Water : Acetonitrile : *o*-phosphoric Acid [67 : 32 : 01 v/v/v] with a flow rate of 0.8 ml/min. The UV absorbance of eluent was monitored at a wavelength of 240 nm.

total duration of 90 days. Distribution of treatment is as follows:

Group – I: PS 250 mg BID (38 subjects)
Group – II: Placebo 250 mg (microcrystalline cellulose 124 mg + lactose 124 mg + magnesium stearate 2 mg) BID (37 subjects)

Both the PS and placebo capsules were white opaque, Size 1, and looked identical.

Inclusion/exclusion criteria

Eligibility was based on the following inclusion criteria: Clinically examined normal healthy volunteers, devoid of any chronic, organic or dreadful diseases, irrespective of religion and income status with age between 45 and 55 years, not taking any supplements or vitamins and male subjects. The exclusion criteria were volunteers of age below 45 years and above 55 years, any chronic, organic or dreadful diseases, concomitant serous disorders, other drug treatment being received simultaneously on long basis which may affect the study results, any immunosuppressive drugs being received, poor nutritional status, subjects likely to fail compliance with the trial protocol, alcoholism, smoking, using antipsychotic drugs and steroids/male contraceptives.

Observation criteria

Screening and diagnosis

Ageing men often present with a variety of vague nonspecific symptoms that may be associated with testosterone deficiency (Nandy *et al.*, 2008). Subjects were, therefore, screened on the basis of the St Louis University ADAM

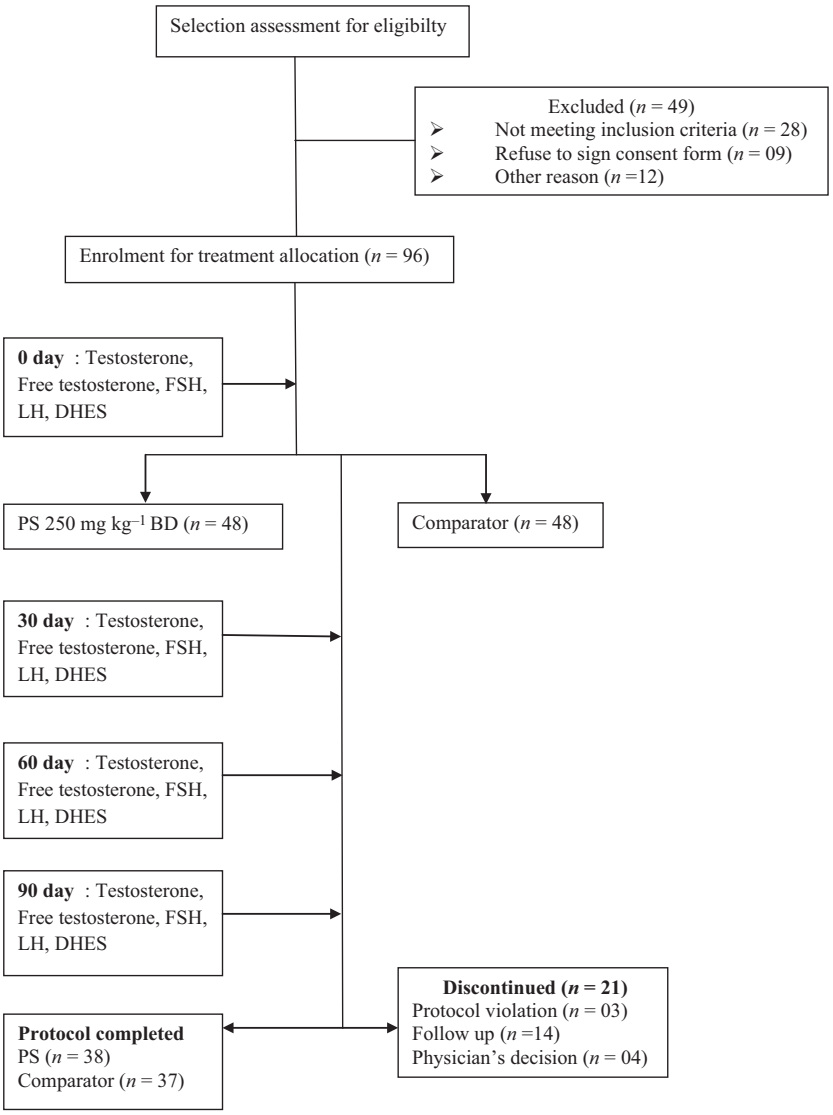

Fig. 2 Schematics of study design.

questionnaire (Table 1) (Morales *et al.*, 2000). Besides the ADAM questionnaire, normalcy of the subjects was established after receiving acceptable ranges of different haematological and biochemical parameters such as fasting glucose, serum urea, creatinine, ALT, AST, Hb%, total RBC, total and differential counts of WBC, RBC/WBC morphology, ESR and routine stool examination including occult blood test. Biochemical investigations were performed by means of auto-analyser (Beckman Coulter, CA, USA), and haematological investigations were done by means of automated cell counter (Medonic CA 530 16; Oden (Merck, Germany). Routine stool examination was carried out by means of conventional methods.

Estimation of testosterone, free testosterone, LH, FSH and DHEAs

Both total and free testosterones, LH, FSH and DHEAs were estimated from fasting blood of each volunteer on days 0 (baseline), 30, 60 and 90. LH and FSH were estimated by means of ADVIA Centaur XP Immunoassay Systems (SIEMENS, Berlin, USA) with ADVIA Centaur LH ready pack primary reagent; testosterone and DHEAs were estimated by means of Access Immunoassay System (Beckman Coulter) with Access Testosterone Reagent Pack and Access DHEAs Reagent Pack respectively. Free testosterone was estimated by means of AccuBind ELISA Microwells Test system (Monobind Inc., Lake Forest, CA, USA) with AccuBind free testosterone reagent pack.

Statistical analyses

Statistical analysis of data of the two treatment groups collected at different intervals of study was performed by paired Student's '*t*-'test using SPSS11.5 version software (Chicago, USA).

Table 1 The St Louis University ADAM questionnaire for screening of testosterone deficiency

Sl	Questionnaires	Comments
1	Do you have a decrease in libido (sex drive)?	Yes / No
2	Do you have a lack of energy?	Yes / No
3	Do you have a decrease in strength and/or and endurance?	Yes / No
4	Have you lost height?	Yes / No
5	Have you noticed a decreased 'enjoyment of life'?	Yes / No
6	Are you sad and/or grumpy?	Yes / No
7	Are your erections less strong?	Yes / No
8	Have you noticed a recent deterioration in your ability to play sports?	Yes / No
9	Are you falling asleep after dinner?	Yes / No
10	Has there been a recent deterioration in your work performance?	Yes / No

Test is considered positive if answers are 'Yes' to question 1, question 7, or any 3 other questions.

Results

Subject screening

All subjects responded 'yes' to questions 1 and 7 or any three other questions according to the ADAM questionnaire were primarily selected. Besides, they also fulfil all inclusion criteria. Fasting glucose, renal investigations like serum urea and creatinine, hepatic investigations like ALT and AST, haematological parameters and stool investigations were found within normal range during primary screening for the inclusion of the volunteers.

Treatment efficacy

A total of 21 subjects discontinued the study, and any data from these subjects were not included in the calculations as these subjects discontinued at different time points of the study and for various reasons.

Testosterone and free testosterone estimation

It was observed that in PS-treated group, there was an increase in testosterone levels (ng ml^{-1}) on days 30 (6.82%), 60 (3.09%) and 90 (20.45%) with respect to day '0'. The increment of testosterone levels on day '90' was significant ($P < 0.05$) with respect to the values of day '0'. In placebo-treated group, there was a significant ($P < 0.05$) decreasing trend of testosterone level. The level of testosterone in PS-treated group on day 90 was found to be significantly ($P < 0.05$) better than the values of placebo-treated group in same day (Table 2). The level of free testosterone (pg ml^{-1}) in PS-treated group on day 90 (19.14%) was significantly better ($P < 0.05$) than 0 day value and maintain parity with the testosterone level. Free testosterone level of PS-treated group on day 90 was also found to be significantly higher ($P < 0.05$) with the values of placebo-treated group on same day (Table 2).

LH and FSH estimation

LH (mIU ml^{-1}) and FSH (mIU ml^{-1}) are inter-related hormones, which have role in synthesis and release of testosterone. In the present research work, it was observed that there was maintenance of LH level in PS-treated group, while FSH level significantly increased ($P < 0.004$) in PS-treated group on days 30, 60 and 90 with respect to baseline. The result of FSH was significantly better in PS-treated group than placebo group on day 90 (Table 2).

Table 2 Effect of PS on testosterone and its mediators with respect to placebo control

	PS (250 mg BID) (n = 38)				*Placebo* (250 mg BID) (n = 37)			
Parameters	Baseline	30 days	60 days	90 days	Baseline	30 days	60 days	90 days
Testosterone (ng ml^{-1})	4.84[b] (1.54)	5.17 (1.33)	4.99 (1.41)	5.83[a,b] (1.67)	5.82[b] (1.58)	4.88[a] (1.74)	4.58[a] (1.44)	4.45[a,b] (1.78)
Free Testosterone (pg ml^{-1})	15.36[b] (7.17)	14.20 (3.97)	14.14 (3.59)	18.30[a,b] (7.72)	19.30[b] (5.75)	15.03[a] (4.22)	14.52[a] (6.19)	12.21[a,b] (5.39)
LH (mIU ml^{-1})	6.33 (3.88)	6.65 (3.95)	6.64 (3.81)	6.79 (3.67)	6.49 (3.32)	7.82 (5.71)	5.86 (2.66)	7.45 (5.90)
FSH (mIU ml^{-1})	6.94 (3.52)	8.17[a] (4.15)	8.52[a] (4.41)	8.41[a] (4.61)	6.91 (6.35)	8.11 (6.06)	7.46 (5.53)	10.23 (11.77)
DHEA-S (μg dl^{-1})	145.09 (53.17)	158.35 (63.56)	159.00 (79.56)	190.57[a,b] (73.24)	139.60 (63.18)	136.04 (68.65)	150.92 (76.08)	138.77[b] (74.17)

$P < 0.05$ considered as significant level in paired and unpaired Student's t-test.
[a]Compare to 0 day value of same group.
[b]Compare to placebo group.

DHEAs estimation

DHEAs, the precursor of testosterone, showed interesting results with PS, where the level of DHEAs (μg dl^{-1}) gradually increased on day 30 (9.14%), 60 (9.59%) and 90 (31.35%) with respect to values on day '0'. Change of DHEAs in placebo group was irregular. However, the level of increase in DHEA in PS-treated group on day 90 was found to be significantly higher ($P < 0.05$) than baseline value of PS-treated group and 90-day value of placebo (Table 2).

Discussion

Testosterone is an anabolic steroid synthesised primarily by the Leydig cells in the testes in males, the ovaries in females and adrenal glands in both sexes. It is synthesised from cholesterol, with androstenedione, androstenediol, dehydroepiandrosterone sulphate (DHEAs), progesterone and pregnenolone acting as some of the intermediate substrates. Testosterone production is regulated by hormonal secretions from the hypothalamus and the pituitary gland in the brain via hypothalamus–pituitary–testicular axis. The process begins as the hypothalamus secretes gonadotropin-releasing hormone (GnRH) in generative pulses. In response to these steady intermittent bursts of GnRH, the pituitary gland releases luteinising hormone (LH) and follicle-stimulating hormone (FSH), which act directly on the testes. FSH activates the Sertoli cells that produce sperm (spermatogenesis). LH stimulates the Leydig cells to secrete testosterone in a daily rhythm characterised by peak levels in the morning and low levels in the evening. Once it reaches high levels, testosterone production generates negative loop feedback to the hypothalamus to downregulate LH release and diminish further testosterone production. In this way, testosterone inhibits its own secretion (Gingrich, 2010). In addition to hypothalamic influence, it has been found that testosterone has direct negative feedback effects on the anterior pituitary gland. Like most hormones, testosterone is supplied to target tissues in the blood where much of it is transported bound to a specific plasma protein, sex hormone-binding globulin (SHBG) (Brooks, 1975). Most circulating testosterone is bound by SHBG and albumin; approximately 2% of total testosterone is free (not bound to protein). SHBG-bound testosterone is so tightly bound that it is not biologically active. Both free and albumin-bound testosterones are biologically active and together are referred to as the bioavailable fraction (Bhasin *et al.*, 2010). It is reported that the total, free and bio-available testosterone varies widely in the age group between 18–69 and 70–89 (Rosner *et al.*, 2007). In the current study, total and free testosterones were estimated to establish the efficacy of PS for its role on testosterone stimulation and secretion property considering the reference range 4–30 pg ml^{-1} for free testosterone and 1.75–7.81 ng ml^{-1} for testosterone (total testosterone). DHEAs, the main precursor of testosterone, was also estimated to rationalise the role of PS on testosterone synthesis considering reference range 70–495 μg dl^{-1} between the age group of 41–50 years and 38–313 μg dl^{-1} between the age group of 51–60 years. The present research work reveals that PS may be able to increase both total testosterone and free testosterone with respect to baseline value indicating its potentiality on testosterone secretion level. The significant betterment of DHEAs with the treatment of PS signifies its role on testosterone synthesis. Other two gonadotropic hormones, *viz.* LH and FSH, were studied in this present work, to rationalise the hypothalamo-pituitary–testicular axis, where both of these hormones were in maintained levels indicating their initial role of triggering of testosterone production. This was followed by downregulation of LH and FSH on one hand and maintenance of the hypothalamo-pituitary–testicular axis by means of elevated level of testosterone on 30, 60 and 90 days on the

other hand. All these modus operandi of PS on synthesis and stimulation of testosterone are found to be better than placebo-treated group in healthy male volunteers in age group of 45–55 years, who may undergo andropause in normal course. Placebo, composed with microcrystalline cellulose, lactose and magnesium stearate, has neither stimulation nor inhibiting role on testosterone secretion or synthesis.

Processed Shilajit (PS) containing biologically active component di-benzo-alpha-pyrone (DBP) is earlier reported to increase the spermatogenic activity in selected patients of oligospermia (Biswas *et al.*, 2009), and the current study demonstrates that purified Shilajit increases the total and free testosterone levels in healthy volunteers.

Conclusion

The present study was conducted to evaluate the efficacy of PS for testosterone secretion and stimulation effects on normal healthy volunteers in the age group of 45–55 years. This effect was clarified by estimation of free and total testosterone on 0, 30, 60 and 90 days where the rise of these two androgenic markers was significant. Testosterone synthesis and secretion was supported by the maintenance levels of two gonadotropic hormones LH and FSH as well as elevation of testosterone precursor DHEAs.

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