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Clinical evaluation of spermatogenic activity of processed Shilajit in oligospermia

T. K. Biswas[1], S. Pandit[1], S. Mondal[1], S. K. Biswas[1], U. Jana[1], T. Ghosh[1], P. C. Tripathi[2], P. K. Debnath[3], R. G. Auddy[4] & B. Auddy[4]

1 Research Unit, J. B. Roy State Ayurvedic Medical College and Hospital, Kolkata, India;
2 Institute of Postgraduate Ayurvedic Education and Research at SVSP Hospital, Kolkata, India;
3 Department of Kayachikitsa, J B. Roy State Ayurvedic Medical College and Hospital, Kolkata, India;
4 Natreon Inc., Salt Lake City, Kolkata, India

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Correspondence
Tuhin Kanti Biswas, Research Unit, J. B. Roy State Ayurvedic Medical College and Hospital, 170-172, Raja Dinendra Street, Kolkata-700004, India.
Tel.: +91-33-25335019;
Fax: +91-33-23371910;
E-mail: biswastuhin@rediffmail.com

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Summary

The safety and spermatogenic activity of processed Shilajit (PS) were evaluated in oligospermic patients. Initially, 60 infertile male patients were assessed and those having total sperm counts below 20 million ml^{-1} semen were considered oligospermic and enrolled in the study ($n = 35$). PS capsule (100 mg) was administered twice daily after major meals for 90 days. Total semenogram and serum testosterone, luteinising hormone and follicle-stimulating hormone were estimated before and at the end of the treatment. Malondialdehyde (MDA), a marker for oxidative stress, content of semen and biochemical parameters for safety were also evaluated. Twenty-eight patients who completed the treatment showed 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%) with concomitant decrease in pus and epithelial cell count compared with baseline value. Significant decrease of semen MDA content (−18.7%) was observed. Moreover, serum testosterone (+23.5%; $P < 0.001$) and FSH (+9.4%; $P < 0.05$) levels significantly increased. HPLC chromatogram revealed inclusion of PS constituents in semen. Unaltered hepatic and renal profiles of patients indicated that PS was safe at the given dose. The present findings provide further evidence of the spermatogenic nature of Shilajit, as attributed in Ayurvedic medicine, particularly when administered as PS.

Introduction

The art of living depends upon several factors including maintenance of heredity with healthy progeny. Healthy progeny depends upon healthy gametes from both male and female partners of conjugated life style. Current epidemiological evidence suggests that 15% of couples in the world experience infertility and half remain untreated and/or unresolved. The distribution of the main diagnostic groups for infertility due to the male is 25%; ovulation 25%; tubal 20%; unexplained cause 25% and endometriosis 5% (Templeton, 1995). Among infertile couples, 40% are primarily due to the infertility of the male partner, while in 20% of these cases it is a combination of both male and female factors that lead to infertility (Agarwal & Kulkarni, 2003). Out of several causes of male infertility, in clinical practice oligospermia is considered one of the most prevalent causes (Haslett *et al.*, 2002).

Literally, oligospermia means insufficient number of spermatozoa; but scientifically it means a medical symptom characterised by less than 20 million spermatozoa per millilitre of ejaculate (http://en.wikipedia.org/wiki/Oligospermia, 2008). The basic pathophysiology of oligospermia is still unknown but several hypotheses are considered to be responsible for oligospermia. Follicular stimulating hormone (FSH) and luteinising hormone (LH) play an important role in spermatogenesis. LH primarily stimulates Leydig cells to secrete testosterone; testosterone and FSH

are the two hormones that act directly on Sertoli cells to promote spermatogenesis. Synthesis of androgen-binding protein is a FSH-dependent process and this protein binds testosterone and dihydrotestosterone and thus provides a local androgenic pool to support gametogenesis (Pramanik, 2007). Therefore, the levels of testosterone and FSH act as marker components and their deficiency may result in oligospermia.

Partial mechanical obstruction may also result in oligospermia, which will lead to imminent surgical intervention (Berek *et al.*, 1988). The excessive generation of reactive oxygen species (ROS) by abnormal spermatozoa and contaminating leucocytes has been defined as one of the important aetiologies for male infertility. Generation and persistence of ROS in seminal fluid and sperm increase the rate of lipid peroxidation of the sperm membrane, which is manifested by a high malondialdehyde (MDA) level (Maneesh & Jayalakshmi, 2006); a decrease in such high MDA levels of semen undeniably favours spermatogenesis.

There is no known specific drug for the management of oligospermia in modern medicine. Extensive clinical research is going on in oligospermia utilising various natural sources of plants, mineral and animal origin as mentioned in different classical traditional texts throughout the world, including Ayurveda, the Indian ancient system of medicine. Shilajit is considered one of the wonder medicines of Ayurveda, which since ancient times, has been utilised for the management of male reproductive disorders.

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), *pahar-ki-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 disease 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). Several toxicological studies, both acute and sub-chronic, have already been performed by many scientists with Shilajit throughout the world. Per oral LD_{50} was found to be >2000 mg kg^{-1} (Acharya *et al.*, 1988; Ghosal *et al.*, 1989) and Shilajit proved to be safe at doses of 0.2 g kg^{-1} and 1 g kg^{-1} when used chronically (Kelginbaev *et al.*, 1973; Anisimov & Shakirzyanova, 1982; Fortan & Acharya, 1984; Al-Hamaidi & Umar, 2003).

Traditional uses of Shilajit primarily focus not only on diabetes and diseases of the urinary tract, but also 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 *Vrsya* (an aphrodisiac with special reference to spermatogenesis) (Sharma 1998).

However, despite its wide-spectrum use as an aphrodisiac by traditional Ayurvedic practitioners, no scientific report has so far been obtained for the spermatogenic activity of Shilajit in oligospermia in a clinical setting. This research study was aimed at exploring the scientific evaluation of Shilajit for its spermatogenic activity in oligospermic patients. In addition, evaluation of safety was carried out in the same Shilajit-treated oligospermic patients.

Patients and methods

Preparation and analysis of the drug

Crude Shilajit rock was procured from Indian Herbs, Saharanpur, India; a voucher specimen is kept in the laboratory with the specific code number SJ/01/05. The processing of Shilajit was carried out by a patented procedure (Ghosal, 2002): crude Shilajit rock was pulverised and passed through a 40-mesh screen. Powdered samples were extracted with hot (60 °C) water, maintaining a solid: solvent ration of 1 : 6, for 1 h. It was filtered and the process repeated once again with the marc. The final pooled filtrate was spray dried to make fine free-flowing powder, which is dark brown in colour and designated as processed Shilajit (PS). The powder was stored in desiccators at room temperature (24 ± 2 °C). PS powder (100 mg) was formulated in hard gelatin capsules. The standardisation of PS involved application of various analytical and chemical methods like HPLC, HPTLC, FT-IR, 1H-NMR, UV-Vis Spectrophotometric examination, GC-MS, ESR spectroscopy. HPLC was carried out in a WATERS (USA) HPLC system with PDA detector and isocratic mobile phase consisting of acetonitrile: orthophsophoric acid: water (32 : 1 : 67) with a flow rate of 0.6 ml min^{-1} using C-18 Novapak reverse phase column attached with a guard column for separation. The injection volume was 20 μl in water. The photodiode array detector wavelength was set at 240 nm. Particle size of the Shilajit in solution was analysed using evaporative light scattering instrument.

Selection of patients

The clinical trial was conducted between June 2006 and January 2008 at J. B. Roy State Ayurvedic Medical College and Hospital, Kolkata, India, after obtaining necessary permission from Institutional Ethics Committee (IEC) and the protocol was approved by the Scientific Review Committee (Ayurveda), Government of West Bengal. The IEC did not permit inclusion of any placebo group in this study considering the grave nature of the disease. Patients who had a history of primary and secondary infertility for a period of 1–5 years were initially selected for the study. Thorough and relevant investigations were performed prior to inclusion and those matching inclusion and exclusion criteria were admitted to the study. Other relevant physical features were also considered. A total of 60 male patients aged between 30–45 years were initially included from the outpatient department of the same hospital. Of these, 21 men were excluded as they did not meet the inclusion criteria, two patients did not sign the consent form and two patients did not start the treatment schedule. The remaining 35 patients were enrolled for the treatment after obtaining consent according to the WHO Helsinki protocol.

Inclusion/exclusion criteria

Eligibility was based on the following inclusion criteria: infertile male patients aged between 30 and 45 years, irrespective of religion, occupation, income status, sperm count below 20 million sperm per millilitre, average motility <60%, normal sperm <65% and spermia <3.0 ml, not currently receiving any other treatment from outside and willingness to give written informed consent for participation in the study. The exclusion criteria were any concomitant serious disorders of vital organs, receiving or having received any infertility treatment for the past 1 month and any other treatment being received simultaneously that may influence the study. Patients suffering from azoospermia, asthenospermia, necrospermia/necrozoospermia and teratospermia/teratozoospermia were also excluded from this study. Patients with a history of mumps, measles, small pox, chicken pox, tuberculosis or injury to genitalia in the preceding 10 years were also excluded from the study.

Safety study

Safety evaluation was also carried out during the same study period, in the same group of patients with the same dose of PS (100 mg twice daily). Additional 2 ml of blood was collected for this purpose at the beginning and at the end of 90 days of PS treatment.

Treatment schedule

All patients were treated with PS 100 mg twice daily after principal meals, which were continued for 90 days to each patient. The present dose of PS (200 mg day^{-1}, p.o.) has been determined from the recommended dose of Shilajit in most of the debilitating states of health (Halpern, 2003) which also simulated with less than 1/10th of LD_{50} dose in animals (Ghosal *et al.*, 1989). Treatment of each patient was monitored weekly. No other treatment was advised to any patients that may influence the study. Each patient was closely observed for any adverse effects with PS. Subjects were given 15 days worth of capsules at each visit and compliance was monitored by traditional pill-count method at each follow-up visit and at the end of the study. A schematic representation of the study design is given in Fig. 1.

Observation criteria

Semenogram is considered to be the direct method of analysis for the diagnosis and prognosis of oligospermic patients. This was performed using the Giemsa and Leishman staining technique (Sood, 1999). Semen of each patient was collected for this purpose after a period of complete abstinence for not less than 7 days. The features observed under the semenogram included measurement

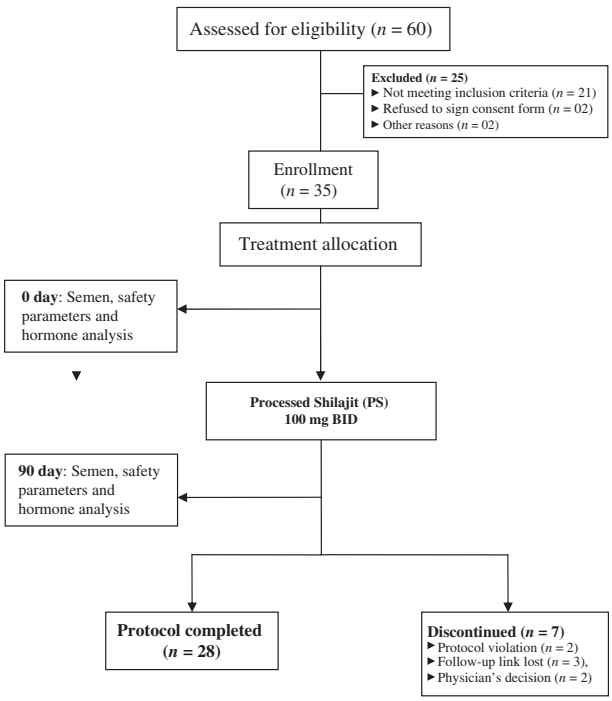

Fig. 1 Schematics of study design.

of spermia, seminal pH, total sperm count (million ml^{-1}), liquefaction time (minutes), motility after 30, 60 and 120 min, sperm morphology study (% of normal sperm), pus cells (per field), epithelia cells (per field) and red blood cells (RBC) (per field). These were estimated on day '0' and day '90' interval. Haematologically, haemoglobin concentration (mg dl^{-1}), RBC (morphology), white blood cells (WBC) (cmm), platelet count ($\times 10^5$ per ml) and erythrocyte sedimentation rate (ESR) (mm h^{-1}) were measured following standard methodology (Briggs *et al.*, 2003). These examinations were performed for the exclusion of any concomitant acute and chronic diseases and were estimated at '0' and '90' days of treatment with the help of an automated cell counter (Sysmex KX-21, Kobe, Japan).

The amount of MDA in the total semen was measured as an index of lipid peroxidation. This was determined using the thiobarbituric acid (TBA) assay (Mihara & Uchiyama, 1978). Briefly, 100 μl of reconstituted lyophilised semen was added to 400 μl phosphate buffer (100 mM, pH 7.4) and 1 ml of TBA reagent containing 0.8% TBA (Sigma, St. Louis, USA), 15% tri-chloro acetic acid, 25% (1 M) HCl and 0.4% butylated hydroxytoluene in distilled water. The samples were heated in a boiling water bath for 30 min, cooled and centrifuged at 700 ***g*** for 10 min and absorbance of the supernatant was measured at 532 nm. All values were expressed as nmoles MDA per mg of dry semen.

In endocrinological investigations, serum testosterone, LH and FSH levels were estimated on day '0' and days '90' of each patient to evaluate the role of the test drug on the hormonal level. These hormones were estimated by means of an electrochemiluminescence immunoassay using a fully automated immunoanalyser, ELECSYS 1010 (Roche, Germany) and Beckman Coulter reagent kit (Villepinte, France).

The safety aspect of PS for the management of oligospermia was assessed on the basis of different biochemical parameters: fasting blood sugar (mg dl^{-1}), urea (mg dl^{-1}), creatinine (mg dl^{-1}), uric acid (mg dl^{-1}), total bilirubin (mg dl^{-1}), total protein (gm dl^{-1}), albumin (gm dl^{-1}), globulin (gm dl^{-1}), SGOT (U ml^{-1}), SGPT (U ml^{-1}) and alkaline phosphatase (U l^{-1}). These parameters indicate the renal and hepatotoxicity of the test substances with specificity and systemic toxicity in general. Biochemical parameters were estimated using an automated analyser (Hitachi-902/BS-300; Japan).

Chromatographic analysis of semen was carried out to assess the presence of PS bioactives in semen. This innovative investigation helped us to identify another important marker for assessment of the effect of PS in spermatogenesis. Semen samples collected from each patient at the commencement and after 90 days of PS treatment were used for this purpose. The samples were lyophilised within an hour of ejaculation and stored at −20 °C. The lyophilised semen samples were dissolved in double distilled water at a concentration of 5 mg ml^{-1}, sonicated for 10 min, followed by centrifugation for 10 min at 4 °C at 1250 ***g***. The chemical constituents of the supernatant were analysed by HPLC using the conditions mentioned before. The PS (0.5 mg) was similarly dissolved in 1 ml of water (with sonication) and analysed by HPLC.

Statistical analysis

Percentage changes for measures were expressed as the difference between means of the baseline and treatment phases divided by the mean of the baseline phase multiplied by 100. Paired Student's *t*-test was carried out with the initial and final values and $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the computer statistical package SPSS/10.0 (SPSS, Chicago, IL, USA).

Results

Standardisation of processed Shilajit

Standardised PS according to HPLC analysis (Fig. 2) was found to contain bioactive components like free and conjugated di-benzo-alpha-pyrones (DBPs), DBP-chromoproteins (DCPs) and fulvic acids and its equivalents within specified limits as mentioned in Table 1.

Treatment efficacy

The objective variables were estimated on the basis of semenogram, haematological parameters and endocrinological investigations. Semenogram investigation revealed that there was a significant ($P < 0.001$) increase of spermia (37.6%) and total sperm count (61.4%), and a reduction of pus cell (−55.5%) and epithelial cells (−81.1%). Significant ($P < 0.001$) gradual increase of sperm motility was also observed after 30 min (12.4%), 60 min (13.2%) and 120 min (17.4%) interval. Per cent increment in normal sperm (18.9%) was also found to be significant ($P < 0.001$) after 90 days of treatment with PS at a dose of 100 mg twice daily. Significant increment ($P < 0.001$) of haemoglobin (5.2%) after 90 days of treatment was observed indicating normal maintenance of body physiology of patients. This was supplemented with a significant increase ($P < 0.05$) of WBC (6%). The most important biochemical markers for spermatogenesis like testosterone, LH and FSH were also estimated and a highly significant increase

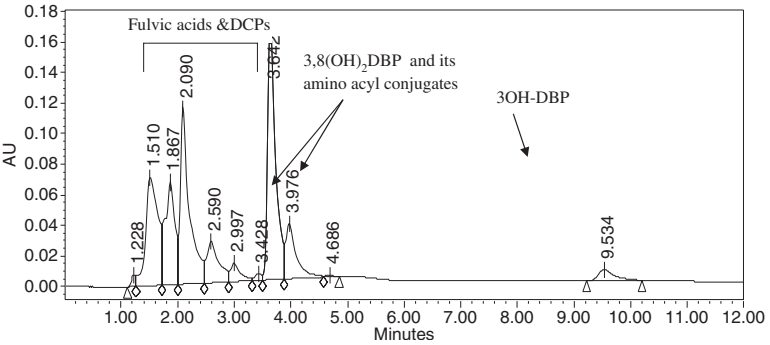

Fig. 2 HPLC chromatogram of processed Shilajit by RP-C18 column.

Table 1 Bioactive constituents with specified limits of processed Shilajit

Bioactive constituents	Specification (%; w/w)	Actual content (%; w/w)
Free and conjugated di-benzo-alpha-pyrones (DBPs)	≥0.30	0.45
DBP-chromoproteins (DCPs)	≥10.00	16.75
Fulvic acids and its equivalents	≥50.00	54.80

($P < 0.001$) of testosterone (23.5%) and significant ($P < 0.05$) increase of FSH (9.4%) were observed (Table 2). However, no significant alteration was observed in serum LH level of the PS-treated patients. A significant decrease (−18.7%, $P < 0.001$) in semen MDA content was also observed in patients who had received PS treatment.

HPLC analysis of semen

A distinct difference in HPLC chromatogram of the semen samples was observed before and after 90 days of treatment with PS (Fig. 3a,b). Several small peaks denoting Shilajit constituents (3-OH DBP, 3,8$(OH)_2$DBP and its amino acyl conjugates) were incorporated in the semen after treatment.

Table 2 Objective variables at baseline and after 90 days of treatment with processed Shilajit 100 mg BID) in oligospermic patients (n = 28)

Parameters	Processed Shilajit (100 mg BID)		
	Baseline	90 days	% change
Semenogram			
Spermia (ml)	2.13 (0.72)	2.93 (0.94)	37.6[a]
pH	7.90 (0.22)	8.00 (0.22)	1.27
Total sperm count (million ml^{-1})	10.60 (4.34)	17.11(8.97)	61.4[a]
Liquefaction time (min)	38.71 (19.35)	39.33 (11.73)	1.60
Motility			
After 30 min	64.07 (20.1)	72.03 (22.7)	12.4[a]
After 60 min	59.64 (20.1)	67.53 (21.8)	13.2[a]
After 120 min	51.36 (19.1)	60.32 (20.1)	17.4[a]
Morphology (% of normal sperm)	62.79 (14.2)	74.68 (6.4)	18.9[a]
Pus cells (per field)[c]	5.46 (3.2)	2.43 (2.5)	−55.5[a]
Epithelial cells (per field)[d]	1.32 (1.2)	0.25 (0.4)	−81.1[a]
RBC (per field)	NF	NF	NC
Haematology			
Haemoglobin (mg dl^{-1})	12.80 (1.14)	13.46 (0.90)	5.2[a]
RBC (morphology)	Normocytic	Normocytic	NC
Platelet count (× 10^5 per ml)	1.56 (0.2)	1.62 (0.3)	3.8
WBC (cmm)	6650 (1170)	7050 (1268)	6.0[b]
ESR (mm h^{-1})	12.2 (6.5)	10.9 (7.8)	−10.7
Endocrinology			
Testosterone (ng ml^{-1})	4.85 (0.82)	5.99 (1.08)	23.5[a]
LH (mIU ml^{-1})	7.45 (2.98)	7.33 (2.23)	−1.6
FSH (mIU ml^{-1})	8.94 (3.78)	9.78 (3.74)	9.4[b]
Oxidative stress level			
MDA (μmol mg^{-1})	0.091 (0.02)	0.074 (0.02)	−18.7[b]

Values represent mean (SD); [a]$P < 0.001$, [b]$P < 0.05$, [c]Pus cells were present in 26 patients and [d]Epithelial cells were present in 20 patients.

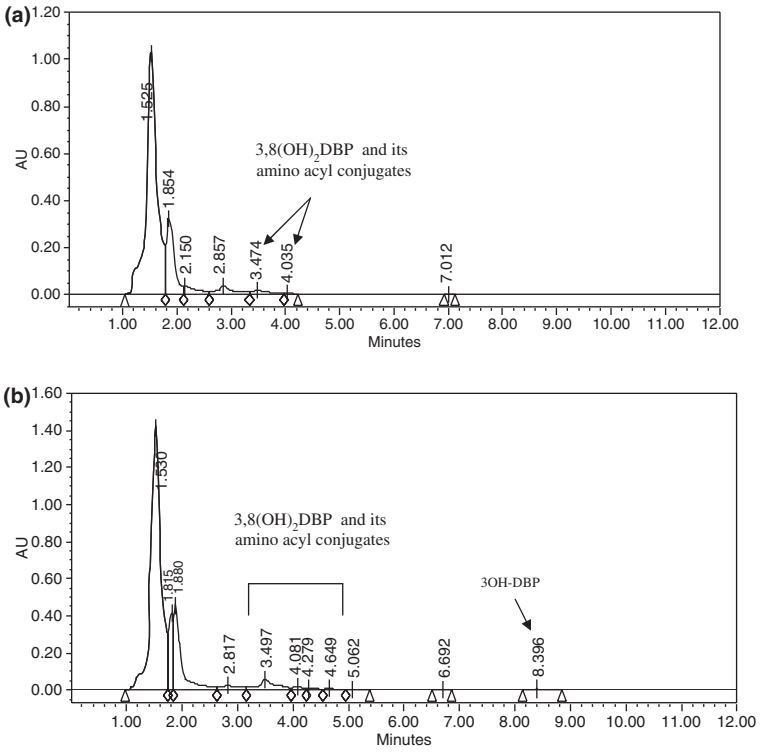

Fig. 3 (a) HPLC analysis of semen on day 0. (b) HPLC analysis of semen after 90 day treatment with processed Shilajit.

Safety

Investigations on the safety of PS at a dose of 100 mg twice daily revealed that there was no alteration in any of the objective features related to any systemic toxicities like serum urea (−0.9%), uric acid (−7.5%), serum bilirubin (9.6%), total protein (−1.3%), serum globulin (−6.7%), SGPT (7.5%), SGOT (−3.5%) and alkaline phosphatase (−6.7%). Significant lowering ($P < 0.05$) of fasting blood sugar (−6.8%) supports the hypoglycaemic effect of Shilajit (Trivedi *et al.*, 2004) and serum creatinine (−7.8%) indicates that the drug does not show any adverse effect in renal profile even in normal state (Table 3). Twenty-eight patients completed this study. There were seven drop-out cases. The main reasons for study withdrawals were protocol violations, follow-up link lost and physician's decision (Fig. 2). No treatment-emergent adverse events were reported to the doctors by any patients during the treatment period.

Table 3 Safety parameters at baseline and after 90 days of treatment with processed Shilajit (100 mg BID) in oligospermic patients (n = 28)

	Processed Shilajit (100 mg BID)		
Parameters	Baseline	90 days	% change
FBS (mg dl^{-1})	95.6 (9.7)	89.1 (7.4)	−6.8[a]
Urea (mg dl^{-1})	21.1 (5.0)	20.9 (3.8)	−0.9
Uric acid (mg dl^{-1})	5.3 (1.4)	4.9 (1.5)	−7.5
Creatinin (mg dl^{-1})	0.90 (0.14)	0.83 (0.11)	−7.8[a]
Total bilirubin (mg dl^{-1})	1.04 (0.33)	1.14 (0.39)	9.6
Total protein (gm dl^{-1})	7.6 (0.6)	7.5 (0.4)	−1.3
Albumin (gm dl^{-1})	4.7 (0.4)	4.7 (0.3)	NC
Globulin (gm dl^{-1})	3.0 (0.6)	2.8 (0.4)	−6.7
SGPT (U ml^{-1})	36.1 (14.2)	38.8 (15.1)	7.5
SGOT (U ml^{-1})	34.1 (10.5)	32.9 (8.5)	−3.5
Alkaline phosphatase (U l^{-1})	129.8 (42.7)	121.1 (41.0)	−6.7

Values represent mean (SD); [a]$P < 0.05$.

Case study

Four oligospermic patients informed about their fertility after 3 months of treatment with PS (100 mg twice daily):

Case 1: TKK, 37 years, a serviceman, complained of infertility after 3 years of married life. Investigations indicated that his wife had no problems to conceive. The overall health status of the patient was good. Semenogram of the patient showed low spermia (1.5 ml), low sperm count (15.5 million ml^{-1}) and presence of pus cells in semen; serum testosterone level was found to be in the lower range (4.77 ng ml^{-1}). After 3 months of treatment with PS (100 mg twice daily), spermia and sperm count increased to 2.5 ml (66.7%) and 22 million ml^{-1} (41.9%) respectively. No observable pus cell was detected in semen and serum testosterone level increased to 6.53 ng ml^{-1}.

At the same visit, he mentioned about his wife's amenorrhoea for the previous 1 month and he confirmed the initiation of pregnancy with laboratory reports.

Case 2: SS, a 30-year-old farmer, complained of infertility after 5 years of married life. His wife maintained normal menstrual cycle. The patient had no history of complicated disease and possessed an overall healthy status. Semenogram of the patient showed low spermia (2.0 ml) and low sperm count (17.6 million ml^{-1}); presence of pus cells and epithelial cells in semen and serum testosterone was found to be in the lower level (3.85 ng ml^{-1}). After 3 months of treatment with PS (100 mg twice daily) the spermia and sperm count increased to 3.0 ml and 22.5 million ml^{-1} respectively. No observable pus cell and epithelial cell were detected in semen. Serum testosterone level was increased to 4.71 ng ml^{-1}. He also mentioned about the amenorrhea condition of his wife for the last two cycles and in the follow-up visit he confirmed his wife's initiation of pregnancy along with laboratory reports.

Case 3: TSG, a 36-year-old businessman, complained of anxiety, hyperacidity, loss of strength, diminished sexual activity and mentioned that he had no issue after 3 years of married life. The patient possessed an overall good health. Semenogram of the patient showed low sperm count (13.5 million ml^{-1}), slightly lower sperm motility (65 after 1 h) and presence of pus cells in semen. Serum testosterone level was initially 3.59 ng ml^{-1}. After 3 months of treatment with PS (100 mg twice daily) distinct improvement in sexual activity was reported with concomitant decrease in anxiety and impending doom. The sperm count also increased to 27.7 million ml^{-1}. No observable pus cell was detected. Serum testosterone level was increased to 4.99 ng ml^{-1}. He also mentioned about the amenorrhea condition of his wife for the last one cycle and in the follow-up visit he confirmed his wife's initiation of pregnancy along with laboratory reports.

Case 4: SS, a 32-year-old information technology professional, communicated with the principal investigator of this study. The patient was suffering from infertility for 3 years. The patient possessed an overall health status and had no other history of complicated disease. Semenogram of the patient showed low sperm count (19.5 million ml^{-1}), slightly lower sperm motility (60% after 1 h) and a high concentration (59%) of abnormal sperm forms. Pus cells were also observed in the semen. After 3 months of treatment with PS (100 mg twice daily) the sperm count increased to 37.1 million ml^{-1}. Sperm motility was increased to 80% after 1 h and normal sperm cell count increased to 60%. Only a few pus cells were detected in semen. Serum testosterone level increased from 3.80 to 5.01 ng ml^{-1}. His wife conceived and this was subsequently confirmed within 1 week of withdrawal of treatment, indicating that the pregnancy ensued during the treatment period.

Discussion

The male partner is considered a primary cause for infertility amongst couples and oligospermia is one of the most prevalent reasons for male infertility in clinical practice. In most of the cases, functional deformity in spermatogenesis is the major reason for oligospermia, which involves either defective mechanism of testosterone, LH and FSH secretion, or there may be excess ROS production, or both. ROS can have beneficial or deleterious effects on sperm cells depending on their concentration (Murawski *et al.*, 2007). Under normal physiological conditions, spermatozoa produce a small amount of ROS for proper capacitation and acrosomal reaction. ROS in semen are generated mainly by neutrophils and also by abnormal spermatozoa (Griveau & Le Lannou, 1997). Excessive production of free radicals or ROS can damage spermatozoa because their plasma membrane and cytoplasm contain a large amount of polyunsaturated fatty acids (Agarwal *et al.*, 2003). It has already been well documented that high levels of ROS in the semen (seminal plasma + spermatozoa) induced lipid peroxidation and were negatively correlated with the quality of spermatozoa in the semen (Alvarez & Storey, 1995). MDA content of semen acts as a major marker for lipid peroxidation.

In this study, it was clearly envisaged that PS could potentially control oxidative stress, which is reflected by lowered MDA levels and may stimulate spermatogenesis (Gomez *et al.*, 1998; Jeong *et al.*, 2006). This evidence is supplemented by significant increment of both testosterone and FSH, the two marker hormones that can directly induce spermatogenesis (Table 2). At physiological level, testosterone did not produce any inhibition of FSH secretion, thus concomitant increase in serum levels of these hormones, observed in this study, can promote spermatogenesis. Higher level of testosterone, in turn, inhibited LH secretion, which was also reflected in this study. Apart from spermatogenesis, testosterone also controls the functional competence of the accessory sex organs, such as prostate gland and seminal vesicles. Adequate seminal fluid, the secretions of seminal vesicles and prostate gland, is necessary for the survival and motility of spermatozoa. Hence, the increased spermia, normal sperm count and motility in the present findings may be due to the higher levels of testosterone. Spermatogenic activity of Shilajit has also been reported pharmacologically in rats where the number of spermatozoa in the epididymis and testes was significantly higher than that in the control animals. The serum testosterone level was also found to be significantly high in Shilajit-treated rats. The changes

of serum LH and FSH were negligible compared with those of the control animals (Jeong *et al.*, 2006).

One of the important constituents of PS is DBP; the ROS generated by spermatozoa themselves and also by the phagocytes of the ejaculate can be put to balance or equilibrium by the reductants present in DBP-conjugates/DBPs (Ghosal, 2006). Other bioactives of PS, DCPs, fulvic acids, its equivalents and amino acids can attenuate the deficiencies in oligospermic patients by captivating ROS and reactive nitrogen species (RNS), provide energy for sperm motility and therefore contribute to sperm health. Chromatographic analyses of semen after treatment with PS indicated inclusion of its constituents in semen. It was observed that PS in solution remains in a fine particle state with average diameter of the particle size being 954 nm. In this state, ingredients of PS are quickly absorbed through the intestinal tract and once in the systemic circulation, it can penetrate the blood-brain (Kreuter *et al.*, 2002) and blood–testis barrier (Kwon *et al.*, 2008). Thus, PS constituents may be transmitted through blood-testis barrier to reach the target organelles, viz., seminiferous tubules and thereby exert their antioxidant actions. Hence, the observable effect of PS is probably due to the direct effect of its active constituents in the spermatogenesis process.

The role of infection in male infertility is increasingly being recognised in the modern world. The decreased level of pus cells and epithelial cells after Shilajit treatment indicated that Shilajit had inhibited the growth of genital bacteria which are generally resistant to a number of currently recommended antibiotics. In literature, Shilajit has been recommended for genito-urinary diseases (Schepetkin *et al.*, 2002).

Conclusion

The present findings, based on advanced diagnostic and clinical methodologies, support preclinical evidence about the effective medical management of oligospermia with PS. Moreover, PS can be used therapeutically at a dose of 100 mg twice daily without any adverse side-effects. HPLC analysis revealed that PS treatment for 90 days caused incorporation of some of its major constituents in semen. As clinical findings indicated a distinct improvement in the quality of semen of the treated oligospermic patients, it can be assumed that these improvements were partly due to inclusion of PS constituents in the semen. Regaining of fertility in four PS-treated patients opens a window in the treatment of oligospermia with natural products. However, the exact role of these components on testicular function is yet to be established. This trend is expected to be more indicative if the PS treatment is continued for a longer period of time.

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