CLINICAL ASSESSMENT OF HERBAL FORMULATION IN REDUCING OXIDATIVE STRESS CAUSING SPERMATOGENETIC DISORDERS USING THE ROS, D- ROMS AND MDA TESTS

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Abstract

Background: Several Ayurvedic textbooks describe *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. for their anti-inflammatory, antipyretic, antioxidant and sexual properties. After obtaining good results in treatment of male sexual disorders during pre-clinical studies, this clinical trial was taken up to assess the antioxidant properties responsible for spermatogenetic activity of aqueous extract of roots of these two plants.

Methods: The study uses subjective evaluation of primary symptoms, estimation of Testosterone levels, sperm analysis and evaluation of oxidative stress levels for assessing the therapeutic efficacy of research formulation through placebo controlled clinical trials on 80 males having lack of sexual desire and non-satisfactory sexual life divided in four study groups. Group I was the control group while Group II received *Sida cordifolia* Linn., Group III *Glycyrrhiza glabra* Linn. and Group IV was administered both these plants mixed equally. The tests for assessment of oxidative stress levels include the d-ROMs test, ROS determination by ELISA Sandwich kit and the determination of MDA level by ELISA method test.

Results: The three drug treated groups (II, III and IV) showed significantly higher therapeutic efficacy in respect of primary symptoms, Testosterone levels and sperm morphology 7 motility as compared to the control group. The decrease in d-ROM value was 2.01% in Group I while it was 16.61%, 12.56% and 20.87% respectively in Groups II, III and IV. Similarly, % decrease in ROS concentration was 4.15, 4.71 and 4.23 in case of Groups II, III and IV while % decrease in oxidative stress marker MDA was 13.58, 12.65 and 13.89 in these treatment groups as compared to very nominal changes in Group I.

Conclusions: During clinical trial, the three drug treatment groups showed significantly higher therapeutic efficacy as compared to the control group. Among these three groups, Group IV containing both *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. exhibited the highest improvement, followed by Group II containing *Sida cordifolia* Linn. and then Group III containing *Glycyrrhiza glabra* Linn. The results of the clinical study confirm the antioxidant and spermatogenetic action of the research formulations.

Key words: Ayurvedic formulation, *Sida cordifolia* Linn., *Glycyrrhiza Glabra* Linn., clinical trial, antioxidant

1. Introduction

Sexual dysfunction can be caused by a variety of reasons including psychological ones such as depression, stress, fear of sex and anxiety, apart from neurological disorders, stroke, cerebral trauma and Parkinson's disease. Other organic causes include chronic renal failure, hepatic failure, multiple sclerosis, Alzheimer's disease, sleep apnea and chronic obstructive pulmonary disease. Decrease in hormone levels with age, systemic diseases like cancer as well as chronic alcohol abuse and cigarette smoking also adversely affect sexual potency.^[1,2]

All living aerobic cells are routinely exposed to few reactive oxygen species (ROS) but if ROS levels rise, oxidative stress (OS) occurs, which results in higher generation of oxygen-derived oxidants that increase the rates of cellular damage. OS has been shown to be a major cause of male

infertility since a large proportion of infertile men have exhibited elevated levels of seminal ROS. Several forms of sperm DNA damage such as chromatin cross-linking, chromosome deletion, DNA strand breaks and base oxidation are caused by ROS. Moreover, ROS are important in mediating apoptosis by inducing cytochrome c and caspases 9 and 3, which in turn result in a high frequency of single- and double-stranded DNA strand breaks. Hence, as far as male infertility is concerned, seminal OS, sperm DNA damage and apoptosis are interlinked, and constitute a unified pathogenic molecular mechanism.^[3-5]

Sida cordifolia Linn. is a small, erect, downy shrub belonging to Malvaceae family which is known by the name of *Bala* in the Ayurvedic system of medicine. It has been used for treatment of diseases like fever, skin diseases, asthma, cough, muscle and joint pain, swelling, inflammation, urinary infection, heart ailments,

facial paralysis, lack of sexual desire and unwanted weight loss and as a astringent, aromatic, diuretic and tonic compound in Ayurveda. [6-8] Its roots and seeds contain alkaloid ephedrine, vasicinol, vasicinone, β -sitosterol, stigmasterol and N-methyl tryptophan while the leaves of *Sida cordifolia* contain ephedrine and pseudoephedrine. Its pharmacological actions include anti-microbial, antioxidant, anti-inflammatory, hypoglycemic, wound healing, analgesic and hepato-protective activities. [9-11]

Liquorice or *Glycyrrhiza glabra* Linn. is a perineal herb/sub-shrub found in the subtropical and temperate zones which belongs to the Fabaceae family. Its underground stems and roots are used for treatment of hyperacidity, cough, skin and ophthalmic diseases and as a rejuvenator, demulcent, tonic and expectorant. [12-16] Its pharmacological activities are reported to be anti-microbial, anti-viral, hypotensive, hepato-protective, anti-exudative, muscle depressant, hypo-lipidaemic, anti-atherosclerotic, antidiuretic, antiulcer, anti-mutagenic, antipyretic, antioxidant, anti-inflammatory, anti-nociceptive and expectorant. The chief constituent of liquorice is glycyrrhizin, which is present in the form of potassium and calcium salts of Glycyrrhizic acid. Liquorice also contains glucose, sucrose, resins, mannite, asparagines and fats. [17-20]

Hence, an Ayurvedic formulation having equal amounts of roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. was prepared for assessing its impact in improvement of sexual desire and other associated symptoms in male human subjects since both these plants showed similar therapeutic actions. This placebo controlled clinical trial was undertaken after getting significant antioxidant, in-vitro reproductive and spermatogenesis activity in the experimental male rat models along with non-toxic and significant pharmacological efficacy. [9-11, 21-23]

The spermatogenesis action of this research drug was evaluated by subjective evaluation of each patient regarding sexual parameters, changes in Testosterone levels and status of sperm morphology, motility and physical properties before and after the clinical trial. The main aim of the present study was to establish its antioxidant activity by evaluating the reduction in oxidative stress levels through direct measurement of ROS levels in patients. Determination of Reactive Oxygen in blood samples was done using the Reactive Oxygen Metabolites (d-ROMs) Test, the Reactive Oxygen Species (ROS) by ELISA Sandwich kit Test and the Malondialdehyde level (MDA) by ELISA method Test.

2. Materials & Methods

2.1. Collection & identification of plant materials

The roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. were purchased from reputed drug supplier of Burdwan district and authenticated by the Research

Officer, Botanical Survey of India, Howrah, India (REF./NO. BSI/CNH/SF/Tech./2016).

2.2 Chemicals and reagents

Glacial acetic acid (ID: CD3C630227) was obtained from Merck Specialties Pvt. Ltd., Mumbai, Ferrous sulphate (ID: 064963) from Sisco Research Laboratories Pvt. Ltd, Mumbai; N, N-diethyl-p-phenylenediamine (ID: 24855816) and Sodium Acetate (ID: 24899538) from Sigma-Aldrich, Mumbai, while ROS Elisa kit was procured from Elabscience (Lot No: AK0016FEB18047) and Human MDA Elisa kit (coated plate) was procured from Bioassay Technology Laboratory (Lot No: 20160203).

2.3. Preparation of Extracts

The aqueous extract of roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. was prepared following the guidelines of Ayurvedic pharmacopeia. One-part coarse powder of research drug was boiled with four parts of distilled water until the quantity was reduced to one fourth. The residual quantity was filtered and concentrated using the lypholizer instrument and stored in dried form for preparation of zero size capsule having average weight 558.2 ± 3.60 gms and dark brown colour. During standardization, the capsules had an average disintegration time of 2 minute 48 seconds and average dissolution time of 30 minutes.

2.4. Selection of subjects

The clinical study was conducted in the OPD of IPGAER Kolkata after getting approval from the Institutional ethical committee for clinical trials (vide no. SVSP/PG/363/2013 dated 22.3.2013) using male human subjects who had given informed consent following the guidelines of ICMR on biomedical research. 90 male patients of 20-60 years' age group suffering from semen disorder (Sukra Dosh) over past 6 months or more were selected after general examination out of which 80 patients finally completed this study after the prescribed study period of 60 days. The inclusion criteria included lack of sexual desire, difficulty in ejaculation or painful coitus. Patients having history of congenital deformities or malignancy, cardiovascular diseases, uncontrolled hypertension, diabetes mellitus or similar complications were excluded. All subjects were randomly allocated to four equal groups and orally administered the prescribed drug. Group I served as the control group where rice powder was given as placebo while Group II was administered *Sida cordifolia* Linn. (Bala) and Group III was given *Glycyrrhiza glabra* Linn. (Yashthimadhu) as the research drug. Group IV subjects received both Bala and Yashthimadhu mixed together in equal amounts in the capsule during the study period.

2.5. Evaluation of subjective and objective parameters

Subjective evaluation of each patient was done to assess the lack of sexual desire, difficulty in ejaculation and getting tired easily. The changes in Testosterone levels were assessed along with sperm morphology, motility and physical properties before and after the study.

2.6. Evaluation of oxidative stress levels

The analysis of concentration of Reactive Oxygen Species (ROS) in the blood samples of patients was done using the three standard methods detailed below.

2.6.1. Determination of Reactive Oxygen Metabolites (d-ROMs Test)

The derivative of Reactive Oxygen Metabolites (d-ROMs) test is a photometric test for measurement of the concentration of hydro-peroxides (ROOH) in biological samples which are substances that belong to a broad class of Reactive Oxygen Metabolites (ROMs) and act as markers and amplifiers of oxidative damage, produced by the attack of free radicals. The d-ROMs test uses the principle of Fenton's reaction. Hydroperoxides are converted into radicals that oxidize Chromogen (N, N-diethyl-paraphenyldiamine) that can be detected through spectrophotometric procedures by changes in colour and its intensity. 20 µl of Serum with 20µl of FeSO₄ and 20µl of D.E.P.P.D were added and thereafter 1.94 ml of 0.1 M of Acetate buffer was added and incubated for 1 min and vortexed. The absorbance of all samples was taken at 505 nm wavelength in the spectrophotometric methods using the Shimadzu UV-2450 instrument. The results of the d-ROMs test are expressed in arbitrary units, called Carratelli units (U CARR), where 1 U CARR is equivalent of 0.08 mg/100ml of H₂O₂.^[24]

2.6.2. Determination of Reactive Oxygen Species (ROS) by ELISA Sandwich kit

Sandwich-ELISA ROS ELISA kit (Elabscience, Lot No: AK0016FEB18047) has been used in this method for determination of the level of ROS in blood serum. The samples of serum were allowed to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 15 minutes and the supernatant is collected. The micro ELISA plate is pre-coated with an antibody specific to ROS. Standards or clear and transparent samples are added to the micro ELISA plate and combined with the specific antibody. Then the biotinylated detection antibody specific for ROS and Avidin-Horseradish Peroxidase conjugate is added to each micro plate successively and incubated. The substrate solution is also added to each well. Only those wells that contain ROS, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of Sulphuric acid solution and the colour turns yellow. The

optical density (OD) is measured spectrometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of ROS in the sample which is calculated from the standard curve.^[25, 26]

2.6.3. Determination of Malondialdehyde level (MDA) by ELISA method

The body produces oxygen free radicals through the enzyme and non-enzyme systems which can attack unsaturated fatty acid on biofilm leading to lipid peroxidation and form lipid peroxide, such as aldehyde group (MDA), keto-, hydroxyl, carbonyl, etc. Oxygen free radicals cause cell damage not only by peroxidation of polyunsaturated fatty acids in biofilm, but also by decomposition products of lipid hydroperoxide. Detection of the MDA content can reflect the level of lipid peroxidation in cells and reflect level of cellular damage indirectly. MDA in the catabolite of lipid peroxide can react with thiobarbituric acid (TBA) and produce red compound, which has a maximum absorption peak at 532 nm.^[25, 27]

MDA ELISA Kit was purchased from Bioassay Technology Laboratory (Lot no. 20160203). This ELISA method also used the Biotin double antibody sandwich technology to assay the MDA level. Standards and samples were added to each well, pre-coated with MDA monoclonal antibody and incubated. Anti MDA antibodies labeled with biotin was added to unit with streptavidin-HRP and again incubated which forms immune complex. Free components were washed away. Substrate A and B were added consecutively which turned the reaction mixture as blue colored solution and the enzyme-substrate reaction was terminated by addition of Sulphuric acid as stop solution. The optical density (OD) was measured at a wavelength of 532 nm in ELISA plate reader. The OD value was proportional to the concentration of ROS and was calculated from a standard curve.

2.7. Statistical analysis

Individual parameters were expressed as mean ± SEM. The Statistical Package for the Social Sciences (SPSS) for Windows, version 10.0.7 (SPSS Inc., Chicago, IL) was used for all calculations and statistical analysis.

3. Results

3.1. Evaluation of subjective and objective parameters

The decrease in primary symptoms observed during the study is shown in Figure 1. The evaluation of Testosterone levels before and after the study period indicated that while it decreased by 1.81% in case of Group I, it increased by 3.30%, 3.93% and 17.69% in case of Groups II, III and IV respectively. The changes in seminal parameters are shown in Table 1.

Figure 1: Change in primary symptoms during the study period

Table 1: Percentage increase in Seminal Parameters

Seminal Parameter		Group I	Group II	Group III	Group IV
Sperm Count (in millions)		1.17	3.75	2.36	4.25
(% Sperm Morphology	Normal Sperm	1.27	3.36	2.49	3.76
	Abnormal Sperm	-4.69	-11.50	-8.02	-12.66
(% Motility (at 1 st hour)	Rapid progressive	1.22	7.92	7.98	11.66
	Slow progressive	3.85	0.62	0.62	1.33
	Non progressive	-11.41	-17.57	-19.43	-25.00
	Immotile	3.45	-8.56	-0.53	-10.07

values are expressed as mean $\pm$ SEM; n=20

3.2. Reactive Oxygen Metabolites (d-ROMs Test)

The results of the d-ROMs test are detailed in Table 2, which describes the decrease in this oxidative stress marker during the treatment period.

Table 2: Results of Oxidative stress marker d-ROM expressed as U. CARR

	Pre-treatment	Post-treatment	% Decrease
Group I	89.39 $\pm$ 7.64	87.59 $\pm$ 7.15	+2.01
Group II	98.31 $\pm$ 8.22	81.98 $\pm$ 7.28	+16.61
Group III	99.70 $\pm$ 8.05	87.17 $\pm$ 7.87	+12.56
Group IV	104.47 $\pm$ 12.79	82.67 $\pm$ 9.49	+20.87

values are expressed as mean $\pm$ SEM; n=20

3.3. Reactive Oxygen Species (ROS) by ELISA Sandwich kit

The concentration of ROS was evaluated before and after the study period whose findings are shown in Table 3.

Table 3: Results of Oxidative stress marker ROS expressed in ng/ml

	Pre-treatment	Post-treatment	% Decrease
Group I	4.40 $\pm$ 0.09	4.36 $\pm$ 0.09	+0.91
Group II	4.34 $\pm$ 0.04	4.16 $\pm$ 0.06	+4.15
Group III	4.37 $\pm$ 0.10	4.16 $\pm$ 0.11	+4.71
Group IV	4.43 $\pm$ 0.04	4.24 $\pm$ 0.10	+4.23

values are expressed as mean $\pm$ SEM; n=20

3.4. Malondialdehyde level (MDA) by ELISA method

The changes in the MDA levels during the clinical trial period have been elaborated in Table 4 and shown in Figure 2.

Table 4: Results of Oxidative stress marker MDA expressed in nM/ml

	Pre-treatment	Post-treatment	% Decrease
Group I	14.08 $\pm$ 1.25	14.38 $\pm$ 1.62	-2.13
Group II	13.64 $\pm$ 1.04	11.79 $\pm$ 1.50	+13.58
Group III	14.27 $\pm$ 2.13	12.47 $\pm$ 1.65	+12.65
Group IV	12.38 $\pm$ 0.99	10.66 $\pm$ 1.43	+13.89

values are expressed as mean $\pm$ SEM; n=20

Figure 2: Changes in Oxidative Stress Markers during clinical trial

4. Discussions

The incidence of infertility varies between 2.5%–15%, correlating to at least 30 million infertile men globally and has been the primary cause of several emotional, physical, and sociocultural problems. One of the mechanisms proposed for male infertility is related to oxidative stress (OS) since studies have found significantly inferior sperm characteristics in infertile men with high levels of ROS in semen as assessed through chemi-luminescence. [25–28]

Spermatozoa are highly susceptible to the damaging effects of ROS due to high concentrations of unsaturated fatty acids in their cell membrane and cytoplasm, limited antioxidant capacity and DNA repair system. Oxidative stress induces a fast loss of intracellular ATP, resulting in axonemal damage with lower sperm viability, mobility and increased mid-piece structural defects, with deleterious effects on sperm efficacy. Lipid peroxidation of the sperm membrane resulting in intracellular oxidative burden is a key mediator of ROS-induced sperm damage, leading to infertility. Reactive oxygen species adversely affects the DNA integrity in the sperm nucleus by inducing breakage of DNA strands, base modifications, and chromatin cross-linking. Increased ROS along with decreased antioxidant defence results in redox imbalance, reduced sperm motility and sperm DNA damage. [25, 27–30]

During the study period, the Testosterone levels decreased by 1.81% in case of Group I, while it increased by 3.30%, 3.93% and 17.69% in case of Groups II, III and IV respectively. Highly significant decrease between 40% to 100% was observed in case of primary symptoms such as

lack of sexual drive, difficult ejaculation and getting tired easily in Groups II, III and IV as compared to the control Group I. The highest efficacy was noticed in Group IV, followed by Group II and Group III.

The sperm count increased by 3.75%, 2.36% and 4.25% in case of Groups II, III and IV as compared to 1.17% in case of Group I, while the percentage of abnormal sperms decreased by 11.50%, 8.02% and 12.66% in Groups II, III and IV in comparison to 4.69% in Group I. In terms of motility after 1 hour, the percentage of rapid progressive sperms increased by 1.22 in Group I while it increased by 7.92, 7.98 and 11.66 in Groups II, III and IV. Similarly, the percentage of non-progressive sperms registered a decrease of 11.41 in Group I and 17.57, 19.43 and 25.00 in Groups II, III and IV. Therefore, as compared to the control group, all the seminal parameters recorded the highest improvement in case of Group IV followed by Group II and Group III.

The d-ROM values of Group I decreased by 2.01% while those of Groups II, III and IV decreased by 16.61%, 12.56% and 20.87% respectively during the clinical trial. Similarly, the percentage decrease in ROS concentration was 4.15, 4.71 and 4.23 in case of Groups II, III and IV while the percent decrease in oxidative stress marker MDA was 13.58, 12.65 and 13.89 in these treatment groups as compared to very nominal changes in Group I. Hence, while no appreciable changes were noticed in case of the control group in respect of the three main oxidative stress markers, highest therapeutic efficacy was noticed in case of the Group IV followed by Group II and then Group III.

5. Conclusions

During the clinical trial, all the treatment groups showed significantly higher therapeutic efficacy as compared to the control group in respect of the subjective and objective parameters and the three oxidative stress markers. Among the three treatment groups are containing the research drug, Group IV containing both *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. Exhibited the highest improvement in all parameters, closely followed by Group II containing *Sida cordifolia* Linn. as the treatment drug, and the Group III containing *Glycyrrhiza glabra* Linn. The results of the clinical study confirm the antioxidant action and spermatogenic actions of the research formulations.

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