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ORIGINAL RESEARCH ARTICLE EXPERIMENTAL

Efficacy and safety of a herbo-mineral ayurvedic formulation 'Afrodet Plus®' in male rats

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ABSTRACT

Background: Reverse pharmacology for drug development has been highly productive and cost-effective in recent past as it is based on the documented therapeutic effects of plants in ancient texts. Afrodet Plus® is formulated for the treatment of male infertility, which contains ancient herbo-minerals. Its efficacy and safety are validated through this animal study in reverse pharmacology mode. **Objectives:** This study was undertaken to evaluate efficacy and safety of an Ayurvedic formulation Afrodet Plus® in adult male rats. **Materials and Methods:** Twelve male rats (Holtzman) between 8 and 10 weeks of age were randomly selected and animals were assigned to a control and two treatment groups. Dosing was performed daily. Various parameters such as weekly body weight, hematology, serum testosterone levels, epididymal sperm count, and efficiency of Daily Sperm Production (DSP) were evaluated. **Results:** It was found that epididymal sperm count had significantly increased in both low-dose (+27.39%) and high-dose (+40.5%) groups as compared to control group. The DSP also showed an increase of 43.7% at high dose of 180 mg/kg body weight as compared to the control group. An increase in sperm motility and especially progressive motility was observed when evaluated by Computer Assisted Semen Analyzer. Histological evaluation of testicular tissue for spermatogenic index revealed that the index had increased in treatment group as compared to control group. **Conclusion:** This study revealed that oral administration of Afrodet Plus® resulted in significant increase in DSP in the testis along with increase in epididymal sperm count and progressive motility as compared to control group without producing any treatment-related adverse effects. These findings provide the documentary evidence that the use of Afrodet Plus® at 90 and 180 mg/kg body weight is effective and safe for the treatment of male infertility especially to improve sperm count and progressive motility.

Key words: Antioxidant, fertility, herbo-minerals ayurveda, spermatogenic activity

INTRODUCTION

Reverse pharmacology for drug development has been highly productive and cost-effective in recent past as it is based on the documented therapeutic effects of plants in ancient texts. Globally, this approach has generated greater interest in Ayurveda and Indian pharmacology so in this milieu, Afrodet Plus® is formulated for the treatment of male infertility which contains ancient herbo-minerals. Its efficacy and safety are validated through the present animal study in reverse pharmacology mode.

The problems of male infertility and impotency are increasing day by day. The global incidence of couple infertility is estimated to be between 10 and 15%.[1] Male infertility is of main concern for today's health issues. Infertility, defined as the inability to conceive after at least 1 year of unprotected intercourse and affects about 8-12% of all married couples, among which about 50% of infertility in couples is attributed to male factor.[2] In Ayurveda, several plants are claimed to possess aphrodisiac potential.[3] Ayurveda has developed a special branch to deal with infertility problems, known as *Vajikarana*. Ayurveda has the potential to treat male reproductive dysfunction with plant-based remedies along with correct diet.

There are various reports on aphrodisiac and spermatogenic activity of different herbs viz., *Mucuna pruriens*,[4] *Withania*

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Received: 24-Aug-2012
Revised: 8-Nov-2012
Accepted: 20-Nov-2012

Access this article online	
Quick Response Code:	Website: www.jaim.in
	DOI: 10.4103/0975-9476.118706

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somnifera,[5] *Asparagus racemosus*, *Chlorophytum borivilianum*,[6] *Pueraria tuberosa*,[7] *Argyreia speciosa*,[8] *Hygrophila spinosa*,[9] and *Anacyclus pyrethrum*.[10] Earlier study on some of the herbs have successfully validated their role in improving sexual behavior and ameliorating sexual dysfunction in rats.[11] On this context, ayurvedic poly-herbo-mineral drug, Afrodet Plus® is formulated. Along with various herbs, *Yashada Bhasma* (*Zinc*) Bhasma is added to the formulation. Yashada Bhasma has been reported to exhibit antioxidant property by enhancing endogenous antioxidant defense mechanism against free radicals.[12] Zinc, as a component of enzyme complexes, plays a fundamental role of antibacterial activity in seminal plasma.[13] The various herbs used in the formulation of Afrodet Plus® are also mentioned in Ayurvedic treatise such as *Sushruta Samhita* and *Charaka Samhita*. The objective of this study was to determine the efficacy in terms of spermatogenic activity and repeated dose safety of Afrodet Plus® using male rat as an animal model.

MATERIALS AND METHODS

Materials

The test substance "Afrodet Plus®" capsules (Batch no. SC018005/AUS-510) were supplied by Solumiks Herbaceuticals Limited. The formulation was stable at room temperature. Each capsule contains following ingredients:

Ingredients:

- *Mucuna pruriens:* 200 mg
- *Withania somnifera:* 100 mg
- *Asparagus racemosus:* 50 mg
- *Chlorophytum borivilianum:* 50 mg
- *Pueraria tuberosa:* 50 mg
- *Argyreia speciosa:* 50 mg
- *Hygrophila spinosa:* 25 mg
- *Anacyclus pyrethrum:* 25 mg
- *Sida cordifolia:* 25 mg
- *Yashada Bhasma:* 25 mg

These ingredients were processed in *Dioscorea bulbifera, Tinospora cordifolia,* and *Emblica officinalis* and preserved with the help of Methyl Paraben Sodium and Propyl Paraben Sodium. All the other chemicals, reagents, and buffer solutions were of standard laboratory grade purchased from Sigma Aldrich.

Methods

Preparation of test material

For administration in rats, the capsule was opened and the powder from capsule was removed. This powder was suspended in 0.02% Gum acacia for uniform suspension. The doses were prepared daily and used freshly.

Test method authentication and animal husbandry

The study protocol involving animals was reviewed and approved at National institute for research in reproductive health (NIRRH) (study number Institutional Animal Ethics Committee (IAEC)/NIRRH/02-09) by the IAEC prior to the initiation of the study and experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals, India.

Healthy adult Holtzman rats of 8-10 weeks age supplied by our Institutional breeding colony were used for the study. The animals were housed in polypropylene cages. The animals were maintained at the controlled temperature of 23 ± 1°C, humidity of 55 ± 5%, and in a 14 h light/10 h dark cycle. The cages contained autoclaved paddy husk as bedding which was replaced on twice weekly basis. Throughout the study, the animals were provided with soy-free, in-house-prepared rat pellets (consisting of crude protein, fiber, and nitrogen-free extract) prepared at the institute throughout the study and filtered drinking water (purified by Ultraviolet and reverse osmosis), *ad libitum*.

Study design

Twelve adult male Holtzman rats between 8 and 10 weeks of age were randomly selected and the animals were assigned to a control and two treatment groups. There was a control group and two treatment groups of 90 mg/kg body weight (BW) and 180 mg/kg BW concentrations of Afrodet Plus®, respectively. The vehicle used for dosing was 0.02% Gum acacia. Oral dose of Afrodet Plus® was administered to adult male rats, by gavage, over a period of 21 days and controls received 0.02% Gum acacia. Animals in the control group were handled in an identical manner to the test group animals. During the experimentation, clinical observations and weekly body weight were monitored. After completion of dosing, animals were sacrificed and subjected to necropsy. The epididymis was immediately subjected to sperm count and motility analysis.

Clinical signs, mortality, and weekly body weight

The animals were examined for any clinical signs of morbidity, mortality, other cage side observations such as behavior, pyloric erection, salivary, eye, and other mucous secretions were observed daily and changes in body weight were recorded weekly throughout the dosing period.

Blood testosterone levels

Blood was collected on day 7 and on termination, serum was separated, kept at –20°C and the testosterone levels were estimated using ELISA kit available commercially (Diagnostics Biochem Canada Inc.)

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Hematology

At the end of the treatment, the animals were bled from the retro-orbital sinus for clinical pathology assessment which included analysis of various hematology parameters such as Hemoglobin, Packed cell volume, Total red cell count, Total white cell count (WBC), absolute erythrocyte indices, and differential WBC count.

Epididymal sperm count and motility analysis

The cauda epididymis was cut and tubules were dispersed into 5 ml of pre-warmed (at 37°C) Medium 199 (with Hank's salts supplemented with 0.5% w/v Bovine serum albumin (BSA), pH 7.4) and incubated for 10 min at 37°C. A drop of supernatant was then subjected for sperm motility. Epididymal sperm motility analysis was carried out using Computer-Assisted Semen Analyzer (CASA) from Hamilton Thorne Research (Beverly, MA, USA). Various parameters such as sperm motility, sperm velocity, path velocity, and progressive velocity were analyzed.

The above suspension was used to determine sperm count using Neubauer hemocytometer.

Daily sperm production (DSP)

At necropsy, one testis from each male is weighed and frozen; the testis is subsequently thawed and homogenized. Certain specific stages (and steps) of spermatids formed during the process of spermatogenesis (from spermatogonial cells to spermatozoa) survive the homogenization step, and their nuclei were counted to provide the number of testicular homogenization-resistant spermatid heads.[14-16]

- Calculation of DSP

$$\text{DSP} = \frac{\text{Spermatid head count per testis}}{\text{Homogenization Resistant Duration}}$$

For rats, Homogenization Resistant Duration = 6.1[17]

- Calculation of Efficiency of DSP

$$\text{Efficiency of DSP} = \frac{\text{DSP}}{\text{Testis weight}}$$

Histological evaluation of testis for spermatogenic index

At the termination, all the animals were sacrificed with CO_2 asphyxiation and subjected to necropsy. Testis and epididymis were subjected to histology. Qualitative analysis of testicular tissue was carried out for calculating spermatogenic index for respective animal.

The spermatogenic index,[18,19] a semi-quantitative estimate of the sperm producing ability of the testes, is based on the types and percentage of germ cells in each stage present in the seminiferous epithelium. Testicular sections are rated on a standardized scale (0-6), based upon the histological appearance of the spermatogenic cells in the seminiferous tubules throughout one or more cross-sections of the testis. Evaluation of the seminiferous tubules to assign the rating includes an assessment of the types of cells present (and the general impression that they are present in normal ratios) and quantitation of the number of late spermatids (steps 15-19 for rats) present in the seminiferous tubules.

Gross pathology, organ weight, and histopathology

After completion of dosing period, the animals were euthanized using CO_2 chamber and necropsied for the gross evaluation of the various organs. The necropsy also included careful and consistent dissection of various target organs such as heart, liver, spleen, kidneys, intestine, stomach, testis, epididymis, and prostate. Various organs viz., Heart, liver, kidney, Adrenal, Prostrate, Spleen, Brain, Testis, Epididymis, Pituitary, and Seminal Vesicle were weighed for absolute organ weight and calculation of organ weight to body weight ratios (Percent Relative Organ Weight). Finally, the dissected tissues were fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin wax. Sections (5 μ) of these tissues taken on glass slides were stained using a combination of hematoxylin-eosin before observing under a microscope for histopathological evaluations.

Statistical analysis

For all the toxicological evaluations, the results of the treatment groups were compared with those of the control group. Data were expressed as mean ± S.D. and were analyzed by two-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Clinical signs, mortality, and weekly body weight

The animals did not exhibit any treatment-related abnormal behavioral signs and symptoms. There was no mortality during the study. No significant differences were observed in weekly body weights of the animals of the treatment groups when compared with that of the control groups [Table 1].

Table 1: Mean of weekly feed intake (gms)

Week	Control	Afrodet 90 mg/kg	Afrodet 180 mg/kg
1st	433±46.145	428.5±17.916	449±24.138
2nd	419±41.841	420.75±21.093	428.5±28.208
3rd	412.5±61.868	423.25±18.751	428.5±27.502

Values (Mean±SD)

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Blood testosterone levels

At the end of 1st week of treatment, there was increase in testosterone levels as compared to control. However, on the day of sacrifice, i.e., on 21st day, there was a decrease in testosterone levels observed in treatment group as compared to control. Average testosterone levels are given in Table 2.

Hematology

There was no significant difference in various hematological parameters as compared to control and treatment groups. Average hematological values are given in Table 3.

Epididymal sperm count and motility analysis

There was significant increase in epididymal sperm count in both low-dose (+27.39%) and high-dose (+40.5%) groups as compared to control group. Average epididymal sperm count is given in Figure 1. There was increase in motility percent and associated parameters such as progressive motility, percent rapid sperms, and area covered in treated groups as compared to control group. This increase was not statistically significant. Average values for motility parameters are given in Table 4.

Daily sperm production

Efficiency of DSP was significantly increased in high-dose group as compared to control group. The DSP was increased by 43.7% at a high-dose group as compared to the control group. Average DSP values are depicted in Figure 2.

Histological evaluation of testis for spermatogenic index

Histopathology of testicular tissue and epididymal tissue did not show any toxicity-related pathological findings. Qualitative analysis of histology section of testicular tissue for spermatogenic index revealed an increase in spermatogenic index which is indicative of increased sperm production in treated groups as compared to

Table 2: Average testosterone levels in blood (ng/ml)

Parameters	Control	Afrodet 90 mg/kg	Afrodet 180 mg/kg
7th Day	503.56±411.269	721.83±333.082	634.63±497.970
21st Day	1034.56±638.140	829.21±402.527	336.25±199.017

Values (mean±SD)

Table 3: Average of haematological parameters

Parameter	Control	Afrodet 90 mg/kg	Afrodet 180 mg/kg
Haemoglobin (g/dl)	15.300±0.337	15.500±0.816	15.275±0.465
RBC (10^6/cmm)	10.625±0.359	10.8±0.509	10.575±0.262
PCV (%)	52.875±1.263	54.175±4.319	52.850±2.549
MCV (pg)	49.75±2.062	50±1.414	50±1.414
MCH (fl)	14.35±0.545	14.275±0.222	14.45±0.289
MCHC (g/dl)	28.95±0.206	28.65±0.730	28.925±0.471
WBC (10^3/cmm)	8.930±1.275	9.388±0.998	8.540±1.600
Lymphocytes (%)	75.000±9.487	80.000±5.888	84.000±6.272
Neutrophils (%)	20.00±9.00	17.00±6.00	14.00±7.00
Monocytes (%)	1.750±1.258	1.750±0.500	1.500±0.577
Platelets (X103/cmm)	542.250±124.620	702.750±75.447	744.750±44.657

RBC=Total red cell count, PCV=Packed cell volume, MCV=Mean corpuscular volume, MCH=Mean corpuscular hemoglobin, MCHC=Mean corpuscular hemoglobin concentration, WBC=Total white cell count, Values (mean±SD)

Table 4: Computer assisted semen analyser parameters for epididymal sperms

Parameters	Control	Afrodet 90 mg/kg	Afrodet 180 mg/kg
Percent motility (%)	42.00±6.557	72.00±11.136	63.00±15.23
Progressive motility (%)	25.00±4.583	36.33±6.429	33.00±8.00
Percent rapid sperm (%)	38.333±6.658	68.667±12.342	58.00±16.371
Area covered (µm/sq)	28.333±4.428	45.775±19.67	55.425±14.20

Values (mean±SD)

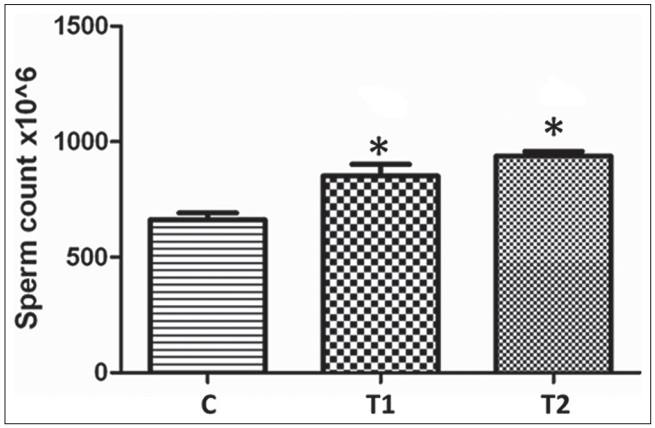

Figure 1: Average epididymal sperm count. x-axis represents epididymal sperm count, y-axis represents treatment groups, data represent Mean ± SD (C-vehicle control, T1-treatment group at 90 mg/kg BW, T2-treatment group at 180 mg/kg BW, *significance 95% ($P < 0.05$))

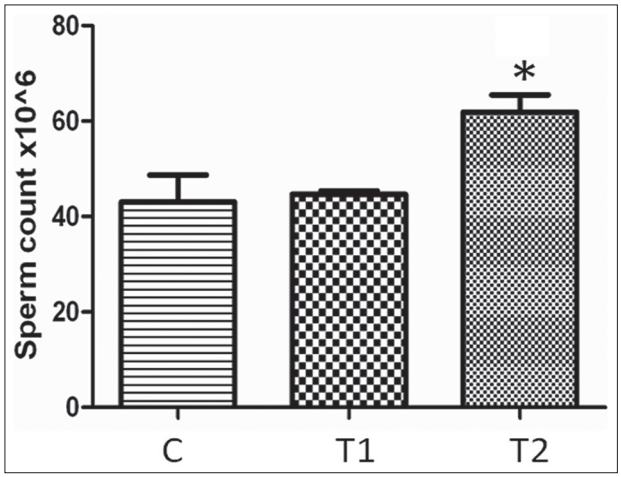

Figure 2: Efficiency of daily sperm production. x-axis represents testicular sperm count, y-axis represents treatment groups, data represent Mean ± SD (C-vehicle control, T1-treatment group at 90 mg/kg BW, T2-treatment group at 180 mg/kg BW, *significance 95% ($P < 0.05$))

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control. The results for spermatogenic index are given in Figure 3 and histology photographs are given in Figure 4. These results are qualitatively indicative of increased sperm production in treatment group as compared to control.

Gross pathology, organ weight, and histopathology

Terminally sacrificed animals did not show any gross pathological or histopathological changes in vital organs. There were no significant difference in absolute organ weights of treated groups and control group except epididymis in Group III (Afrodet 180 mg/kg), where there was significant increase in weight as compared to control. Absolute organ weight is given in Table 5. There were no significant difference in relative organ weights of treated group and control group except epididymis, in Group II (Afrodet 90 mg/kg) and Group III (Afrodet 180 mg/kg), there was significant increase as compared to control. Average relative organ weight is given in Table 6.

Table 5: Terminal body weight and absolute organ weight (gms)

Organs	Control	Afrodet 90 mg/kg	Afrodet 180 mg/kg
Body weight	469.667±39.145	445.500±29.727	458.500±27.197
Heart	1.413±0.139	1.470±0.291	1.463±0.134
Liver	12.553±0.916	16.020±2.536	14.525±1.628
Kidney	2.747±0.047	3.308±0.147	3.208±0.291
Adrenal	0.06±0.003	0.06±0.0129	0.08±0.029
Spleen	0.847±0.154	0.840±0.049	0.983±0.084
Brain	1.988±0.087	1.945±0.147	2.038±0.043
Testes	3.98±0.714	3.848±0.294	4.023±0.199
Pituitary	0.012±0.003	0.013±0.002	0.013±0.004
Prostrate	0.789±0.273	1.11±0.251	0.985±0.042
Seminal vesicle	1.025±0.355	1.138±0.365	0.838±0.144
Epididymis	1.493±0.190	1.780±0.119	2.133±0.409*

*Significance 95% verses vehicle control (P<0.05), Values (mean±SD)

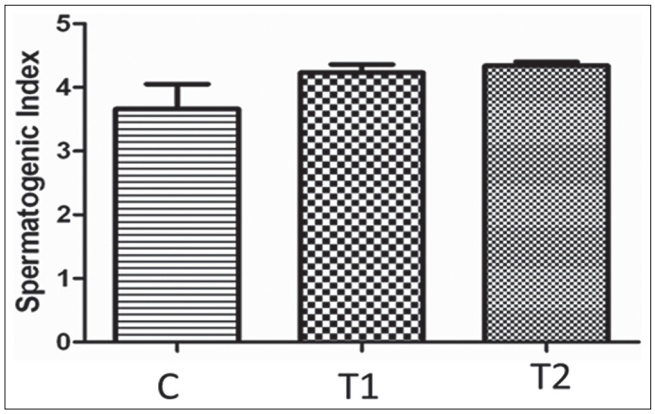

Figure 3: Seminiferous tubule assessments for spermatogenic index. x-axis represents spermatogenic index, y-axis represents treatment groups, data represent Mean ± SD (C-vehicle control, T1-treatment group at 90 mg/kg BW, T2-treatment group at 180 mg/kg BW, *significance 95% (P < 0.05))

DISCUSSION

Afrodet Plus® is a poly-herbo-mineral formulation used as an aphrodisiac and a prophylactic agent in patients with oligospermia to increase the sperm count. It counter acts male sterility and acts as a nervine tonic.[20] *Withania somnifera* improves endurance against stress, thereby helping in management of male sexual disorders such as psychogenic impotence and unexplained infertility.[5,21] Similar kind of findings were reported earlier for *Asparagus racemosus*, *Chlorophytum borivilianum*,[6] *Pueraria tuberose*,[7] *Argyreia speciosa*,[8]

Table 6: Average relative organ weight (%)

Organs	Control	Afrodet 90 mg/kg	Afrodet 180 mg/kg
Heart	0.322±0.018	0.329±0.056	0.319±0.026
Liver	2.689±0.349	3.583±0.402	3.168±0.311
Kidney	0.708±0.119	0.728±0.045	0.699±0.029
Adrenal	0.016±0.009	0.016±0.001	0.018±0.006
Spleen	0.182±0.040	0.189±0.005	0.215±0.023
Brain	0.425±0.039	0.439±0.059	0.446±0.027
Testes	0.863±0.105	0.822±0.201	0.878±0.028
Pituitary	0.003±0.001	0.003±0.000	0.003±0.001
Prostrate	0.157±0.077	0.252±0.074	0.215±0.007
Seminal vesicle	0.216±0.063	0.253±0.069	0.183±0.032
Epididymis	0.319±0.046	0.401±0.042*	0.464±0.072*

*Significance 95% verses vehicle control (P<0.05), Values (mean±SD)

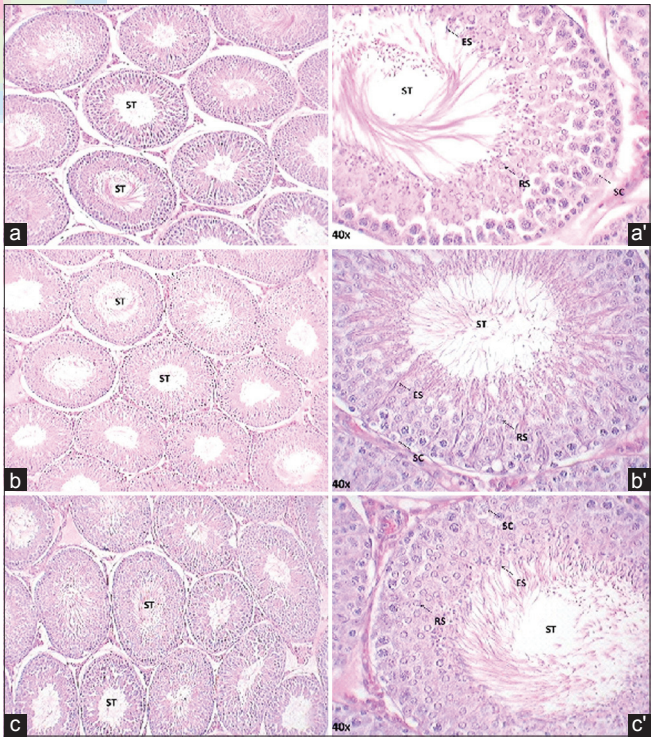

Figure 4: Photomicrographs showing histology of testicular tissue from vehicle control (a, a'), treatment group at 90 mg/kg BW (b, b'), treatment group at 180 mg/kg BW (c, c'). The tissue sections were taken at 4-6 µm and stained with haematoxylin and eosin. (ST-Seminiferous tubules, SC-Sertoli cell, RS-Round Sermatides, ES-Elongated Spermatids)

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Hygrophila spinosa,[9] and *Anacyclus pyrethrum*[10] indicating improved spermatogenesis.

In this study, efficiency of DSP was significantly increased as compared to control group. There was increase in various CASA parameters such as percent motility, progressive motility, percent rapid sperms, and area covered in treated groups as compared to control group. The serum testosterone levels were increased in treatment group as compared to control in first week; however, subsequent levels decreased as compared to the time of termination of the study. Epididymal sperm count and relative and absolute epididymis count were significantly increased as compared to control. The increase in testosterone levels, increase in epididymal sperm count, and increase in weight of epididymis are attributed to *Mucuna pruriens* as it increases testicular testosterone, protein level in epididymis, and epididymal alkaline phosphatase activity.[22] Histological evaluation of testicular tissue revealed that the spermatogenic index was increased in treatment group as compared to control but it was not statistically significant. All of these results were indicative of Afrodet Plus® (helps overcome infertility) primarily acting on epididymal sperm count, as values increased after 21 day of treatment along with an increase in DSP and spermatogenic index. This suggests administration of Afrodet Plus® increases sperm production at given therapeutic doses of 90 mg/kg body weight and 180 mg/kg body weight.

The use of metallic ingredients in ayurvedic formulations has evoked concern and debate in scientific and public forums in recent times. Though Afrodet Plus® contains *Yashada Bhasma* (*Zinc*), there was no significant change in weekly body weight and hematological parameters as compared to control. There was no significant change in relative and absolute organ weight except for epididymis and there was a significant increase in absolute and relative organ weight in treatment group as compared to control. Overall, Afrodet Plus® did not produce any treatment-related adverse effects at high dose (180 mg/kg) in rats.

CONCLUSIONS

The increase in sperm production in treatment group may be attributed to the Afrodet Plus®. It also enhances sperm production at dose 90 mg/kg body weight and 180 mg/kg body weight. Afrodet Plus® did not show any treatment-related adverse effect at high dose of 180 mg/kg in rats. The histological evaluation of heart, liver, and kidney was found to be normal, i.e., no toxicity-related changes were observed. Afrodet Plus® showed significant improvement in the sperm quality, it confirms its therapeutic use in humans. Further, mechanism of action of this drug needs to be elucidated.

ACKNOWLEDGEMENT

Authors acknowledge the assistance provided by Mr. Jayant Tare, Mr. Pravin Salunkhe, Mr. Subhash Kadam, and Mr. Sharad Bhagat. Authors are thankful to Indian Council for Medical Research (ICMR), Department of Science and Technology (DST) and Shree Dhootpapeshwar Pvt. Ltd. for funding the study.

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How to cite this article: Dhumal R, Vijaykumar T, Dighe V, Selkar N, Chawda M, Vahlia M, Vanage G. Efficacy and safety of a herbo-mineral ayurvedic formulation 'Afrodet Plus®' in male rats. J Ayurveda Integr Med 2013;4:158-64.

Source of Support: ICMR, DST, and Shree Dhootpapeshwar Pvt. Ltd., **Conflict of Interest:** None declared.

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