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Biochemical, Pathomorphological and Semen Characteristics Analysis in Male Wistar Rats Treated with *Withania somnifera* Root Extract†

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Withania somnifera (Ashwagandha) is one of the most popular herbal medicines in the treatment of several diseases. Data on the possible toxic effects of *Withania somnifera* over male reproductive parameters are not available. In the present study, male Wistar rats were divided into two groups namely, Group I (control administered with distilled water) and Group II (administered orally with *Withania somnifera* root extract @ 1000 mg/kg body weight for 70 days) of 6 rats each. Blood samples were collected using retro orbital puncture method and various haematological and biochemical parameters were analyzed. No significant changes were found in haematological and biochemical parameters of treated group except for significant decrease in the level of WBC and LYM alone. In the semen characteristics and histopathological analysis, no significant changes were found. The NOEL (No-observed effect level) of *Withania somnifera* Root extract was concluded as 1000 mg/kg in the male wistar rats.

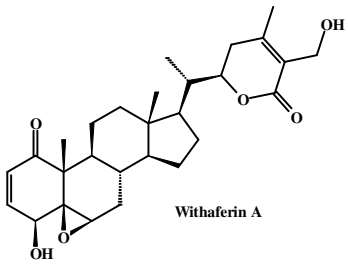
Keywords: Herbal, Ayurveda, Indian Ginseng, Siddha, Alternative medicine, Safety, Reproduction, Toxicity.

INTRODUCTION

Plants and their derivatives play an important role in maintaining health conditions and have been known to possess biological activity and are used as natural medicine from prehistoric times. According to the World Health Organization (WHO), about 80 % of the people living in developing countries depend essentially on medicinal plants for their primary health care[1]. Medicinal plants are widely used in Indian system of medicine and in Chinese medicine. The Indian subcontinent has a vast variety of medicinal plants, approximately in the range of 15,000-18,000[2]. Among the various plants used in Indian system of medicine, *Withania somnifera* (Ashwagandha) is one of the most widely preferred shrubs. It has been used for thousands of years as one of the best remedies in Ayurvedic system of medicine to treat many conditions.

This is probably due to the chemical constituents such as Withaferin present in this plant which possess a wide spectrum of biological activity.

Withanolides (ergostane type steroids) are specific for the genus Withania and are used as marker compounds[5] for chemical characterization. Among them, withaferin A has been studied



Withaferin A

extensively in the past[6] and was used as a phytochemical marker compound for characterization purposes[7]. In spite of the widespread usage, there is a limited data available about the pharmacology and toxicology for the most commonly used herbal remedies[8].

Though, the herbal remedies are considered to be safe, these naturally occurring compounds may also exert a toxic effect in the development or normal functioning of the reproductive system[9-12]. These toxic effects include reduction of male fertility potential by impairing sperm production, maturation, function and survival by acting directly on the sperm in the testis *milieu* or by affecting epididymal function. There is a growing concern about human reproductive disruption by xenobiotics including drugs, occupational and environmental exposures over the past

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decade[3]. Detailed investigations on the different aspects of toxicity studies in appropriate animal models which include sub-acute/sub-chronic/chronic/reproductive/haemtopoietic are essential to assess the possible toxicities associated with the usage of medicinal plants. Such efforts will allow accurate evaluation of changes in animals and shall help in extrapolating human reproductive risk[4]. However, there is only limited literature available on the systemic toxicity of *Withania somnifera.* Hence present study was proposed to evaluate the effect of long term and high dose administration of *Withania somnifera* on the biochemistry, haematology, pathomorphological changes of testes, epididymis and seminal vesicles and semen characteristics in male wistar rats and thereby, to assess the reproductive toxicity associated, following OECD guidelines[13].

EXPERIMENTAL

Hydro-alcoholic root extract of *Withania somnifera* obtained from M/s Ellees aromatics along with certificate of analysis was kept in tightly sealed containers to avoid humid conditions. The plant material was collected (June, 2008) in Neemuch, Madhya Pradesh, India. A voucher specimen was authenticated by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, National Institute of Herbal Science, Chennai and was deposited at the Archives, Central Animal Facility, SASTRA University. The amount of extract needed was taken in small quantities from the stock, as and when required.

General procedure: The animal studies were conducted at the Central Animal Facility, SASTRA University, Thanjavur, Tamilnadu. The experiment was conducted after getting due approval from the Institutional Animal Ethical Committee (IAEC), SASTRA University (IAEC Approval Number: 97/SASTRA/IAEC/RPP). All the animals were examined for any abnormalities, ill health and were weighed. The animals were kept in an identical environmental condition to prevent the influence of external factors. The housing temperature and relative humidity were maintained at 22 ± 3 ºC and 30-70 %, respectively so as to avoid heat/cold stress to the animals. Uniform lighting (12-12 h light/dark) cycle was maintained throughout the study period. All the animals were maintained separately in groups of five in clean and sterile polypropylene cages. The animals were housed in the experimental cages before a week of the actual commencement of experimental study to enable them to acclimatize to the environment. All the animals had access to sterile water and standard laboratory rodent feed *(ad libitum)*. Sterilized paddy husk was used as the bedding material, which was changed on alternate day regularly. All the animals were fed with balanced nutritional rodent feed to avoid any nutritional deficiency. The feed consisted of 4.59 % moisture, 22.30 % crude protein, 3.44 % crude fat, 3.90 % crude fiber, 1.28 % calcium, 0.92 % phosphorous and 6.79 % total ash.

Twelve male wistar rats *(Rattus norvegicus)* of 8-12 weeks of age weighing from 150-300 g and aged between 8-12 weeks were used for this study. After the acclimatization period, the animals were weighed again and assigned to two groups by randomization. Group I was administered with the hydro-alcoholic extract of *Withania somnifera* and the Group II (control) was treated with vehicle distilled water for 70 days. *Withania somnifera* was administered in the dose range of 1000 mg/kg body weight by gavage. The dose to be given for each animal was calculated based on individual animal's body weight and adjusted weekly for changes in body weight. The prepared dosage of the extract of the drug was administered to each animal orally by oral intubation needle. Animals of all the groups were closely observed throughout the experimental period for clinical signs. Body weights of all the animals were recorded on weekly basis. The blood samples were collected by retro orbital puncture method under anaesthesia using microhaematocrit tube/pipette.

Detection method: About 1 mL of blood was collected into dry sterilized tube containing anticoagulant, EDTA for haematological estimations such as WBC, RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelets, mean platelet volume, thrombocrit and platelet distribution width. For biochemical analysis, 1 mL of blood sample was collected and was allowed to centrifuge at 2500 rpm for about 0.5 h. Serum was separated and analysis was done using an automated biochemistry analyzer for parameters such as glucose, total protein, albumin, triglycerides, cholesterol, calcium and creatinine.

The semen characteristics such as sperm morphology and sperm count were analyzed using the following procedures. The animals were sacrificed using CO_2 inhalation and the cauda epididymides (both left and right) was taken out. The cauda epididymides was nicked in several places using a scalpel blade in such a way it extends in to but not through the lumen of the duct and blood vessels are avoided. The tubule segment was immersed in 10 mL of PBS buffer facilitating dispersion in to the buffer which was maintained at 37 ºC. The segment was allowed to disperse for 40 min. After 40 min, 10 µL of each sample was loaded in to a clean glass slide and the smear was taken. It was then dipped in methanol and allowed to dry and proceeded with H and E staining. Sperm morphology was analyzed under light microscopy. For sperm count, 2 mL of PBS was added to 0.5 mL of sample prepared as above and mixed gently, maintained at 37 ºC for 10 min. 10 µL of sample was taken and loaded in the hemocytometer and the spermatozoa were counted under a light microscope. The final sperm count calculation was done using the following formulae:

$$\text{Total count} = \frac{(\text{Mean count} \times \text{dilution factor})}{(\text{Volume of one primary square})}$$

From this, the total count number of sperms/g of epididymis were calculated.

All the internal organs including testis, seminal vesicles and epididymis were thoroughly examined and the gross lesions, if any, were recorded. The weights of the testis, seminal vesicles and epididymis were measured. Small pieces of (1 cm sq) of various organs/tissue were collected from each sacrificed animal and fixed in neutral buffered formalin. After proper fixation of 48 h, tissues were cut into thinner section (2-3 mm thick), processed, sectioned and stained by routine haematoxylin and eosin staining technique.

All the data collected were statistically analyzed to get proper conclusion. The quantitative data of haematological and biochemical parameters, body weight, organ weight and

semen characteristics findings were analyzed by student t-test using WINKS SDA6 Software. All the statements of significance were based on a probability level of 0.05.

RESULTS AND DISCUSSION

The mean ± SE values of the various haematological parameters obtained are given in Table-1. A statistically significant decrease was observed in the white blood cell count and lymphocytes in the treatment group. The value of the monocyte and eosinophil counts showed insignificant decrease in treated group and was comparable to that of the control group. Neutrophil values showed an insignificant increase in treated group and were comparable to that of control group.

TABLE-1
EFFECT OF *Withania somnifera* ROOT EXTRACT ON HAEMATOLOGICAL PARAMETERS OF MALE WISTAR RATS

Parameter	Group I (Mean ± SE)	Group II (Mean ± SE)
WBC (10^3/µL)	7.11 ± 0.36	4.86 ± 0.40*
LYM (10^3/µL)	5.96 ± 0.28	3.73 ± 0.35*
MON (10^3/µL)	0.05 ± 0.02	0.01 ± 0.01
EOS (10^3/µL)	0.06 ± 0.02	0.01 ± 0.01
NEU (10^3/µL)	1.01 ± 0.14	1.05 ± 0.14
RBC (10^6/µL)	8.22 ± 0.16	7.80 ± 0.60
HGB (g/dL)	14.75 ± 0.25	13.66 ± 1.08
HCT (%)	42.16 ± 0.63	39.41 ± 3.15
MCV (µm^3)	51.35 ± 0.51	50.40 ± 0.56
MCH (ρg)	17.95 ± 0.12	17.50 ± 0.15
MCHC (g/dL)	34.96 ± 0.15	34.66 ± 0.14
RDW (%)	14.46 ± 2.58	15.61 ± 0.97
PLT(10^3/µL)	751.83 ± 58.66	652.50 ± 70.25
MPV (µm^3)	5.91 ± 0.06	6.05 ± 0.10
PCT (%)	0.44 ± 0.03	0.39 ± 0.03
PDW (%)	16.05 ± 1.34	19.01 ± 2.00

Means bearing *vary significantly between groups at $p < 0.05$.

RBC counts and PCT values showed insignificant decrease in treated group as compared with that of control group. The RDW value showed insignificant increase in treated group and was comparable to that of the control group. No significant changes in other haematological parameters were found in the treated group as compared to that of control group ($p < 0.05$).

Mean ± SE values of the various biochemical parameters of the control and treated groups are listed in Table-2. No statistically significant changes were observed in the different biochemical parameters of treated group as compared to that of control group (at $p < 0.05$, Figs. 6-8). Glucose, total protein, triglyceride and cholesterol levels were insignificantly lower in treated groups as compared to that of the control group.

TABLE-2
EFFECT OF *Withania somnifera* ON VARIOUS BIOCHEMICAL PARAMETERS IN MALE WISTAR RATS

Parameter	Group I (mean ± SE)	Group II (mean ± SE)
Triglycerides (mg/dL)	127.83 ± 11.80	119.5 ± 19.42
Glucose (mg/dL)	80.50 ± 3.83	68.23 ± 12.96
Cholesterol (mg/dL)	85.33 ± 4.70	83.83 ± 3.19
Albumin (g/dL)	31.36 ± 0.40	41.71 ± 11.06
Protein total (g/dL)	69.03 ± 1.03	67.36 ± 1.48
Creatinine (mg/dL)	1.06 ± 0.02	1.10 ± 0.02
Calcium arzenazo (mg/dL)	14.33 ± 0.30	14.33 ± 0.17

Means bearing *vary significantly between groups at $p < 0.05$.

No significant differences in the body weights, organ weight/relative organ weight of both testes and the seminal vesicles were observed between control and treated groups ($p < 0.05$). The body weight of treated group showed an insignificant increase (433.87 ± 7.03 g) as compared to that of control group (414.96 ± 7.70 g). No significant difference was found in the sperm count and sperm morphology between the treated group and control group (Figs. 1 and 2). The gross pathological and histopathological studies on the samples of both control and treated groups revealed spontaneous background lesion only. No significant treatment related lesions were observed in testes, epididymis and seminal vesicle sections of the treated group (Figs. 3-8).

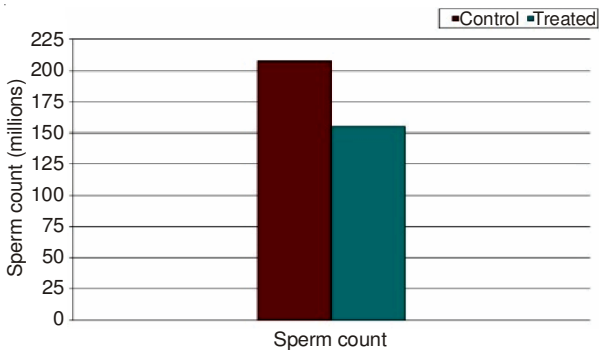

Fig. 1. Effect of *Withania somnifera* on the sperm count in male Wistar rats

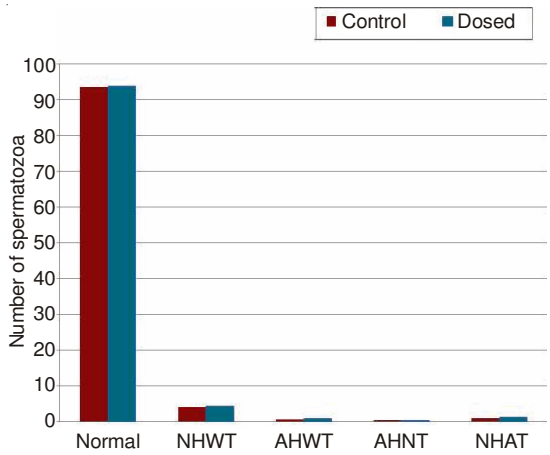

Fig. 2. Effect of *Withania somnifera* on the sperm morphology in male Wistar rats

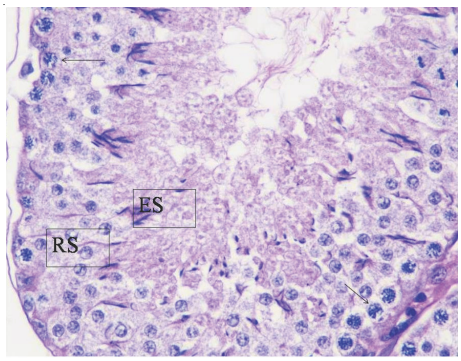

Fig. 3. Testis section from the control rat showing normal histology of the seminiferous tubules with adequate spermatogenic series of cells including spermatocytes (arrow), round spermatids (RS) and elongate spermatids (ES). H& E. 40 ×

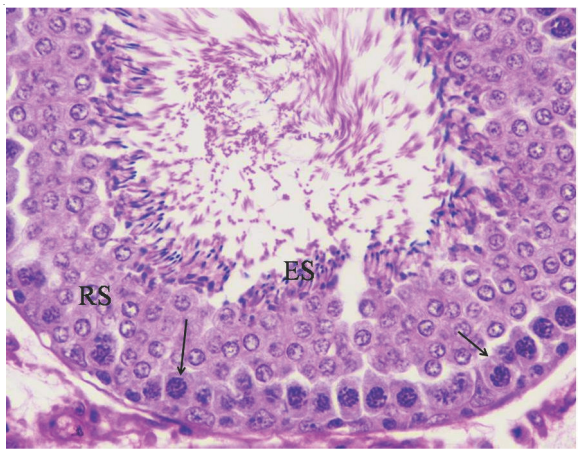

Fig. 4. Higher magnification of the testis section from the WSR root extract treated rat (@1000 mg/kg bw) with no significant pathological changes showing normal spermatocytes (arrow), round spermatids (RS) and elongate spermatids (ES). H& E. 40 ×

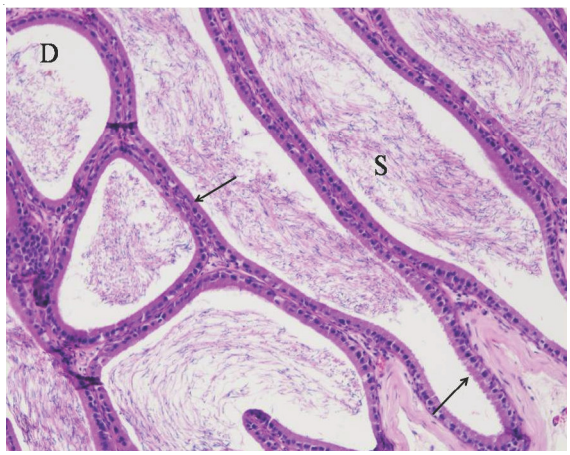

Fig. 5. Section of epididymis of the control rat showing normal ducts (D) lined by low columnar epithelium (arrow) filled with plenty of spermatozoa (S). H&E. 10×

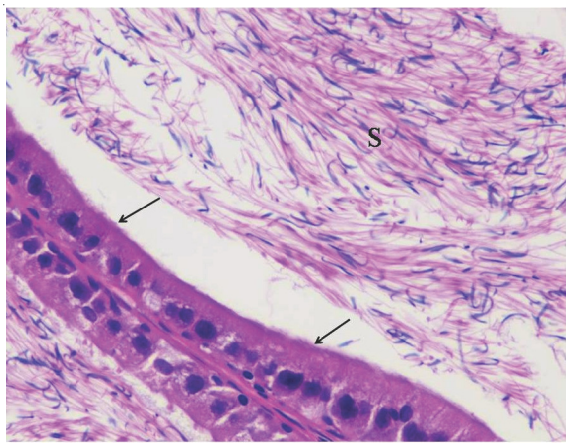

Fig. 6. Higher magnification of epididymis of control showing normal ducts with cuboidal epithelium (arrow) and abundance of spermatozoa (S). H&E. 10 ×

Reproductive capability of the herbal extract under study revealed no significant toxicity over reproductive health and in the development of animals. Shappira and Bhattacharya have reported the improved reproductive capability in both sexes using animal model treated with *Withania somnifera*

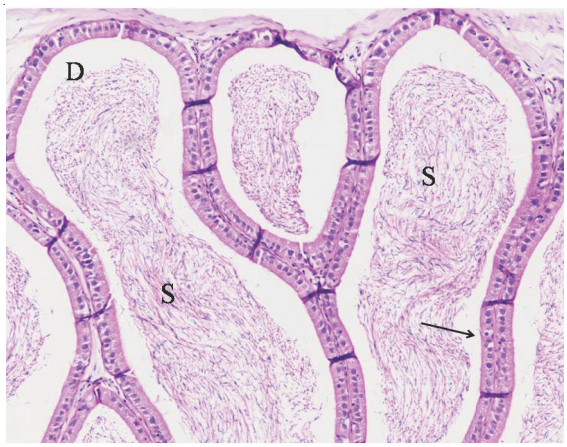

Fig. 7. Section of epididymis of the WSR treated rat (@ 1000 mg/kg bw) showing normal ducts (D) lined by low columnar epithelium (arrow) and numerous spermatozoa (S). H&E. 10×

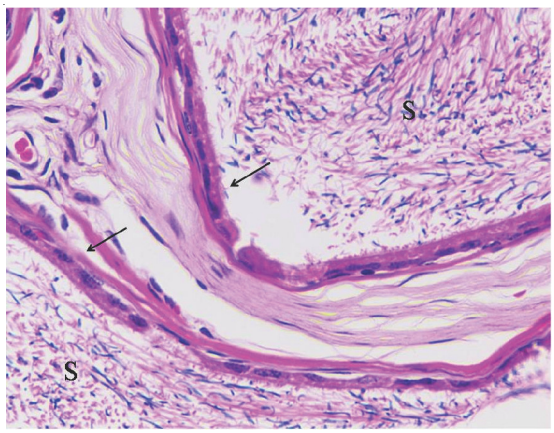

Fig. 8. Higher magnification of section of epididymis of the WSR treated rat (@ 1000 mg/kg bw) showing normal ducts lined by low columnar epithelium (arrow) and numerous spermatozoa (S). H & E. 40 ×

(Ashwagandha)[14,15]. Several studies on this plant have indicated that it possesses anti-inflammatory, antistress, antioxidant properties[16] besides positively influencing the endocrine system[17]. *Withania somnifera* could be one of the major herb contributing to fertility improvements. It could be a combination of direct and indirect effects of this herb to combat stress by pluripotent effecter constituents. Such a potential herb should be proved scientifically as non toxic, which will be a great boom to the suffering human society.

With this objective present study is taken up which revealed that there is no signifcant changes observed in the haematological and biochemical parameters, analyzed revealed no significant changes, except for the levels of leukocytes and lymphocytes in the treated group as compared to that of control indicating that the administration of *Withania somnifera* caused no significant alteration in the overall metabolic picture of the body and its functioning. Besides it is also noticed that long term administration of the extract did not seem to have caused any toxic effect in the rats as evidenced by absence of deaths and other clinical signs of toxicity. The treatment did not cause a significant body weight reduction or interfered with food consumption of the dosed animals that showed similar weight gain when compared to the control. This suggests the non-toxic nature of *Withania somnifera* over body weight and feed

intake. A weight loss of the reproductive organs is under hormonal control and could suggest a disturbance of the reproductive endocrine functions[18]. In the testis, the production of sex steroid hormones and male gametes is regulated by the follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are secreted by the pituitary gland. An impairment of the normal functioning of this gland could, then, interfere with the development and functioning of the male reproductive system[19]. There were no treatment related changes observed on the relative organ weight of testes and seminal vesicle. In this study, the treatment did not produce significant pathological changes in the testes, epididymis, seminal vesicle and coagulating gland as evidenced histologically. Despite the long duration of the treatment, *Withania somnifera* root extract was non-toxic to the testis, the epididymis and the seminal vesicle. In addition to the organ weight data, a comprehensive assessment of the effects of chemicals on male reproductive functioning also requires the study of effects on spermatogenesis which further indicates the quality of spermatozoa[20,21]. A basic understanding of spermatogenesis is essential for the detection of toxicologic effects during preclinical safety studies[22]. In the present study attempts were made to understand the spermatogenesis which revealed no impairment of the spermatogenic cycle, as the sperm concentration from the cauda epididymis did not differ significantly between the control and treated groups. The sperm morphology seemed unaffected by the treatment as the proportion of normal and abnormal spermatozoa was comparatively similar between the control and treated groups. These observations correlated with the histological data that showed no alteration on the histoarchitecture of the testis and epididymis. To sum up the administration of *W. somnifera* extract to the male Wistar rats did not cause significant toxicity as evaluated through hematology, biochemistry, pathomorphology and semen characteristics profiles.

Conclusion

Withania somnifera @ 1000 mg/kg given orally for 70 days did not cause any significant effect in haematology, biochemistry, semen characteristic profile and morphopathology, indicating the absence of toxicity following its exposure at a higher dose in male Wistar rats. *Withania somnifera* @ 1000 mg/kg given orally for 70 days caused a significant decrease in the level of leukocytes and lymphocytes contrary to the previous studies suggesting the need of further investigation, over its effect on immune system.

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