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Protective effect of spermatogenic activity of *Withania Somnifera* (Ashwagandha) in galactose stressed mice

Rahul B. Patil[1], Shreya R. Vora[2] and Meena M. Pillai[3]

[1]*Department of Zoology, Veer Wajekar Arts, Science & Commerce College, Mahalan vibhag, Phunde, Tal.: Uran, Dist.: Raigad, Navi Mumbai (INDIA)*
[2]*Department of Zoology, Shivaji University, Kolhapur (INDIA)*
[3]*Department of Biotechnology, K. I. T.'s Engineering College, Kolhapur (INDIA)*

ABSTRACT

In recent years, population is facing many health problems like chronic non-communicable diseases like Cancer, Stroke, Diabetes, Cardiovascular disorders and Reproductive disorders fro very early age. Urbanization and mechanization have changed the life style of the whole population which has resulted in greater exposure to pollution, high consumption of salt and fat containing food, low physical activity and stress. Withania may be having antioxidant property; protecting male reproductive organs from ROS and can avoid infertility. The overall findings may be helpful to the population not only to treat infertility but also to maintain normal sexual life.

Keywords: *Withania Somnifera*, Antioxidant, Ashwagandha

INTRODUCTION

In recent years, population is facing many health problems like chronic non-communicable diseases like Cancer, Stroke, Diabetes, Cardiovascular disorders and Reproductive disorders fro very early age. Urbanization and mechanization have changed the life style of the whole population which has resulted in greater exposure to pollution, high consumption of salt and fat containing food, low physical activity and stress.

Reproductive failure is observed to be a common problem in young generation due to stress. Sterility and impotence in males is characterized by dramatic change in seminiferous epithelium that leads to decrease in spermatogenesis and steroidogenesis [1] and regression of testis. This is termed as reproductive aging which is caused by increase in oxidative stress, due to overproduction of Reactive Oxygen Species (ROS).

Now a days, people are becoming more and more aware of the use of antioxidant from plants and vegetables to avoid oxidative stress and related health hazards. The antioxidants are reported to prevent oxidative stress caused by reactive oxygen species by interfering with the oxidation process by reactive with ROS, Chelating and catalytic metals and also by acting as oxygen scavengers [2]. Diet rich in antioxidant supplements help to reduce risk of cellular damage due to free radicals. Onions, beet, cabbage, lettuce, parsley, etc. are rich in flavonoids, a class of antioxidants [3].

Apart from these plants, there is presence of many medicinal systems to treat infertility of which Ayurvedic system of medicinal plants is well accepted since long. Many Ayruvedic plants like Shatavari, Brahmi, Amla, Ashwagandha are widely used for their antioxidant properties [4, 5, 6].

In the present investigation, it is decided to study the antioxidant effect of ethanolic extract of *Withania somnifera* (Ashwagandha) on spermatogenesis of oxidatively stressed mice. Oxidative stress will be induced by injected a low dose of D-galactose to male albino mice [7].

Medicinal properties of plants have been investigated throughout the world since long. Recently there has been an upsurge of interest in the therapeutic potential s of plants, as antioxidants in reducing oxidative damage. Suzuki and Sofikitis 1999 [8] have proved that administration of antioxidants enhanced the testicular functions, epididymal sperm motility and fertilizing ability in varicocelized rat. Antioxidants have wide range of different biological activities including antibacterial, antithrombotic, vasodilator, antibacterial, anti-inflammatory and anti-carcinogenic [9]. Flavonol glycosides of Quercetin, Kaempferol and Lutieolin have four times more antioxidant potential than vitamin E. These are suggested to protect against incidences of myocardial infarction [10], DNA cleavage [11, 12] and cognitive deficits [13]. These antioxidants are abundant in red cabbage, Cherries, Parsley and Lettuce.

Thus, apart from the vitamins A, C, E and minerals that act as antioxidants, it has been proved by many researchers worldwide that use of antioxidant rich fresh fruits, vegetables and plants definitely help to lower the risk of oxidative damage in the cells.

India has a proud history of use of Ayurvedic plants, their crude extracts and squash to treat many chronic and acute illnesses. Extensive studies have been carried out to study medicinal properties of *Bacopa monniera* (Brahmi), *Asparagus recemosus* (Shatavari), *Emblica officinalis* (Amla), *Oscimum sanctum* (Tulsi), etc. [14, 15]. Brahmi is well known as brain tonic and extensively used to treat various mental illnesses [16, 4, 5]. Brahmi has also been proved to be effective spermatogenic in induced aged mice [17]. Aphale *et al*, 1998 [18] also stated the importance of phytochemicals present in *Withania somnifera*, as its effect is well studied in female reproductive tract.

The problem of infertility in men is increasing day by day. It has become a source of global concern. The factors such as smoking, drinking of alcoholic beverages, and use of restricted drugs, stress, poor nutrition and lack of exercise play important role in this problem. Again, injury to the testes, blockage in the vas deferens, excessive heat to the testes, vitamin deficiencies and varicocele have also been associated with infertility in men.

Though, there are various treatments for infertility, many diagnostic methods are quite embracing to the patients, which again affect the sexual activity. Scientists have deviced different ways of alienating the problem and one of the easy and cheapest options is herbal medicines. Herbs have been in use since long time to treat various diseases [19].

Withania somnifera is commonly known as Ashwagandha. It is widely used to improve strength and stamina in various ayurvedic preparations [20]. It contains active alkaloids in the form of withanoloids. Ashwagandha was traditionally used to increase vital fluids, blood and lymph. It helps to counter act chronic fatigue, weakness, loose teeth, thirst, premature aging emaciation and convalescence and muscle tension.

Despite many applications o the plant, there is very limited or no information about its efficacy in infertility. Hence, the study is targeted at investigating the effect of ethanolic extract of *Withania somnifera* on testicular function in stressed mice.

MATERIALS AND METHODS

1. **Preparation of Plant Extract:** Fresh leaves of *Withania somnifera* is obtained washed with distilled water and blotted with blotting paper. They were shade dried and crushed to make fine powder. The powder of the plant leaves soaked in distilled alcohol for 72 hours, filtered and evaporated in the vacuum evaporator (Buchi type). The extract was obtained in the form of thick paste. It was collected in glass bottle and stored at 4^0C.

2. **Preparation of groups of male albino mice *Mus musculus* as follows**:
Healthy male mice of 5-6 months age weighing 40- 50gms will be selected and distributed in three groups. Each group will have 4 animals.

Group I: Control group, will receive sterile water 0.5 ml subcutaneously for 15 days.
Group II: Stress induced group, will receive 5 % D-galactose in Sterile water subcutaneously for 15 days.
Group III: Extract co-treated group, will receive 2 % extract of *Withania somnifera* along with D-galactose as group II.

After completion of dosage the animals will be sacrificed by cervical dislocation and testes and epididymis will be excised out to perform

A) Lipid peroxidation [21]:
a. **Total Lipid peroxidation**: The testis and epididymis were weighed, thawed properly and homogenized in reaction mixture (2 mg tissue/ml reaction mixture). Uniformly homogenized mixtures were used as samples for determination of MDA.

b. **Mitochondrial peroxidation**: The tissues were homogenized in 0.25 M sucrose and 1mM EDTA. Centrifugation was carried out at 3000 rpm for 10 min. at 10^0C. The supernatants were again centrifuged at 10,000 rpm. For 10 min. at 10^0C. The supernatants thus obtained were discarded and the pellets were resuspended in 0.2 ml 20% Triton X-100 and 0.8 ml distilled water and centrifuged at high speed (10000 rpm for 10 min. at 10^0C). The pellets obtained after high centrifugation were suspended in reaction mixture and used as samples for the estimation of mitochondrial peroxidation. After additions the tubes were placed in boiling water bath for 10 min. and cooled. After cooling, the samples were read at 532 nm.

Calculations:

$$X = \frac{Ex\ 3x\ 6}{0.156 \times 0.2}$$

Where,
X= amounts of MDA in homogenate n mol/mg tissue
E=absorbance value measured at 532 nm.
3= volume of samples taken for photometric measurement in ml.
0.156= absorbance for 1 n mol solution of MDA measured in 1 cm. thick cell at 532 nm.
0.2= volume of supernatant liquid sample for the determination of MDA in ml.

B) Spermatogenic studies:
1. Sperm count [22]: The sperm count was carried out by using Haemocytometer. Haemocytometer is generally used for RBC as well as WBC count. It is provided with the pipettes for the dilution of the blood samples and Neaubaur's slide with special type of ruling. The counting is done in the ruled squares on the slide.

The epididymis was removed and placed in a pre-chilled Petri-plate. 2 ml. of 0.9% saline was added to it and the epididymis was gently minced with the help of sharp razor. This sample was used for the sperm count. The sample was pipette out with the help of pipette provided in the Haemocytometer. A clean and dry cover slip was kept on the Neaubaur's ruling. The ruling was loaded with the sample by touching the tip of the pipette to the slide. The slide was kept on a bench for 2 min. to allow the sperms to settle down. The sperms were counted in four squares at the corner of the ruling covering an area of 4 sq. mm. under high power objective. The spermatozoa with head and tail were counted.
2. Calculation:

$$\text{Total sperm count/epididymis} = \frac{\text{Sperm count}}{X\ 0.1} \times 1000$$

3. **Histology:** To study the histological structure, the testis and epididymis were fixed in 10% NBF for 24 hours. The tissues were washed under running tap water for 24 hours and dehydrated through alcohol grades, cleared in xylene and embedded in paraffin wax. The sections of 5 micron thickness were cut on a rotary microtome. These sections were mounted on albumenized slides and routinely stained with Heamatoxylin-Eosin (HE).

RESULTS

1. Lipid peroxidation:
a. **Total lipid peroxidation:** Total lipid peroxidation in the form of Malondialdehyde (MDA) n mol/mg tissue wet weight is described in table 1 of testis and epididymis of all groups.
b. It was observed that injection of D-galactose increased the MDA level in both testis and epididymis (23.42±39.60 & 18.30±0.47 n mol MDA/mg tissue) which was about two fold more compared to the control group (9.54±0.34 & 9.30±0.25 n mol MDA/mg tissue). After administration of *Withania somnifera* plant extract, the peroxdation was reduced in testis and epididymis (12.42±0.43 & 9.47±0.26 n mol MDA/mg tissue).

c. **Mitochondrial peroxidation:** Table 2 describes the mitochondrial peroxidation in testes and epididymis of all the study groups.
Peroxidation in mitochondrial fractions of testis and epididymis was significantly increased in D-galactose (4.23±0.085 & 5.10±0.048 n mol MDA/mg tissue) injected group as compared to control one (2.90±0.0025 & 3.10±0.10 n mol MDA/mg tissue). The treatment of plant extract showed decreased peroxidation in testis and epididymis (2.40±0.043 & 2.67±0.025 n mol MDA/mg tissue).

2. Spermatogenic studies:
a. **Sperm count:** Sperm count of all the groups is summarized in Table 3.The sperm count was significantly decreased (5.345±0.0612 x 10^6/epididymis) in D-galactose induced aged mice ($p<0.05$) as compared to control (6.136±0.0835 x 10^6/epididymis) and it was found increased in the Withania administered mice as compared to the galactose treated group (5.520±0.0715 x 10^6/epididymis).

b. **Histology:** The testis of D-galactose induced mice showed degenerative changes in the seminiferous epithelium. Layers of spermatocyte and spermatids were disturbed and ultimately resulted in decreased number of sperms (SP) in the lumen of seminiferous tubules. Leydig cells were observed scattered in the interstitial spaces (L). Thus, the overall structure of seminiferous tubules was found highly disorganized (Plate 1, Fig. 2) as compared to control one (Plate 1, Fig. 1)

The epididymis of galactose injected mice also showed alteration. The epithelial lining of epididymis showed ruptured cells and very few sperms (SP) in the lumen (Plate 1, Fig. 5) as compared to control one (Plate 1, Fig. 4).
The sections of both testis and epididymis receiving *Withania somnifera* along with D-galactose did not showed much degeneration compared to D-galactose. The tubules found normal with no change in the epithelium as well as layers of spermatocyte and optimum spermatozoons in epididymal lumen (Plate 1, Fig. 3 & 6)

Table 1: Effect of *Withania somnifera* extract on Total Lipid Peroxidation of Testis & Epididymis of D-galactose induced aged mice (n mol MDA/mg tissue)

Sr. No.	Study Group	Testis	Epididymis	Statistical significance		
1	Control (5)	9.54±0.34	9.30±0.25			
2	D.G. (5)	23.42±39.60	18.30±0.47	55.45 p<0.001	1:2	33.08 p<0.001
3	D.G.+ *Withania somnifera* (5)	12.42±0.43	9.47±0.26	24.09 p<0.001	2:3	13.78 p<0.001

Number in parenthesis denotes number of animals
Values are Mean±S.D.
P<0.001 highly significant
D.G.: D-galactose injected group
D.G.+ Withania somnifera: Plant extract along with D-galactose injected group

Table 2: Effect of *Withania somnifera* extract on Mitochondrial Lipid Peroxidation of Testis & Epididymis of D-galactose induced aged mice (n mol MDA/mg tissue)

Sr. No.	Study Group	Testis	Epididymis	Statistical significance		
1	Control (5)	2.90±0.0025	3.10±0.10			
2	D.G. (5)	4.23±0.085	5.10±0.048	24.67 p<0.001	1:2	18.45 p<0.001
3	D.G.+ *Withania somnifera* (5)	2.40±0.43	2.67±0.027	89.56 p<0.001	2:3	60.87 p<0.001

Number in parenthesis denotes number of animals
Values are Mean±S.D.
P<0.001 highly significant
D.G.: D-galactose injected group
D.G.+ Withania somnifera: Plant extract along with D-galactose injected group

Table 3: Effect of Withania somnifera on Sperm Count of D-galactose induced Aged mice (Sperm count in x 10^6/epididymis)

Sr. No.	Study Group	Sperm Count	Statistical Significance		
1	Control (5)	6.136±0.0835			
2	D.G. (5)	5.345±0.612	1:2	6.30	p<0.05
3	D.G. + *Withania somnifera* (5)	5.520±0.0715	2:3	2.80	p<0.05

Number in parenthesis denotes number of animals
Values are Mean±S.D.
P<0.05 significant
D.G.: D-galactose injected group

D.G.+ Withania somnifera: Plant extract along with D-galactose injected group

PLATE – I

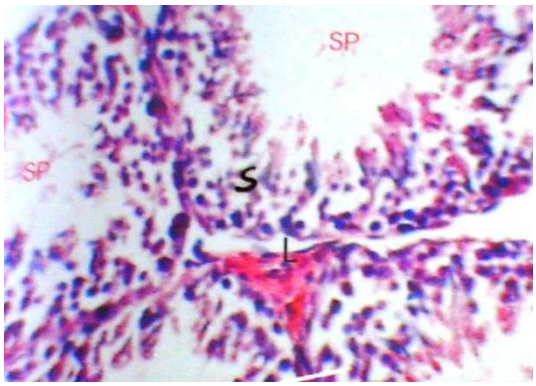

Fig. 1

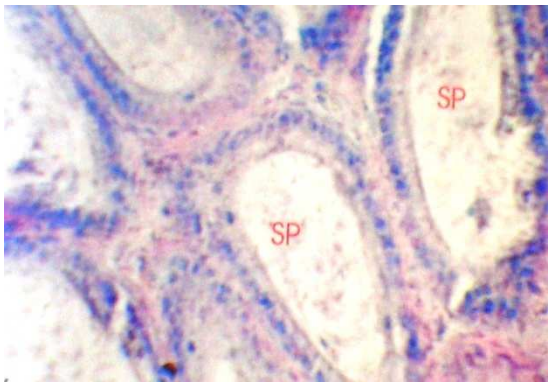

Fig. 2

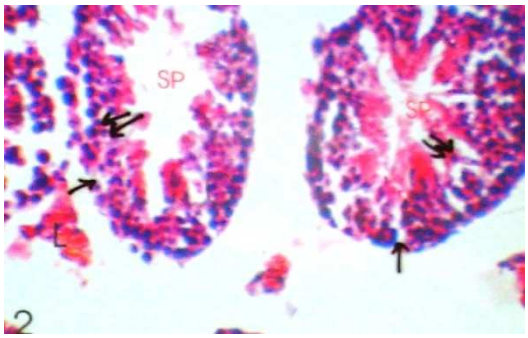

Fig. 3

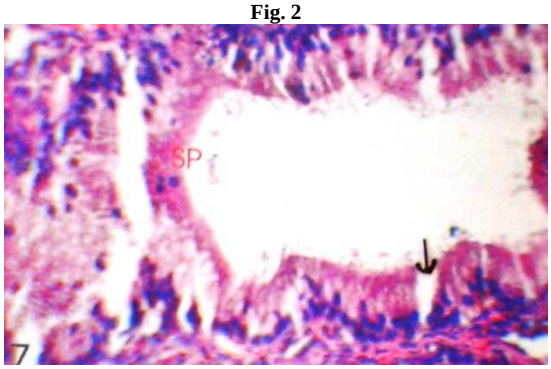

Fig. 4

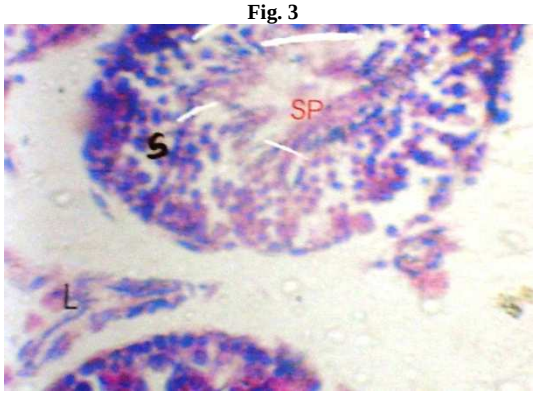

Fig. 5

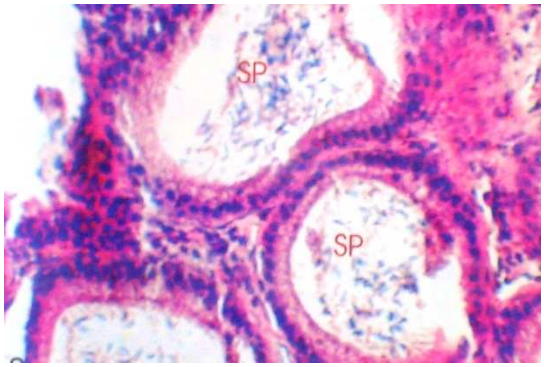

Fig.6

PLATE –I Demonstration of Testicular Cells

Fig.1: Control group of Testis stained with Eosin+Hematoxylene x 128
Fig.2: D-galactose group of Testis stained with Eosin+Hematoxylene x 128
Fig.3: Extract of Withania somnifera along with D-galactose injected group of testis
Fig.4: Control group of Epididymis stained with Eosin+Hematoxylene x 128
Fig.5: D-galactose group of Epididymis stained with Eosin+Hematoxylene x 128
Fig.6:Extract of Withania somnifera along with D-galactose injected group of Epididymis

DISCUSSION

In the present investigation there was increase in the total as well as mitochondrial peroxidation in both testis and epididymis of D-galactose stressed mice as compared to control one. Song DU and his colleague 2002 [23] have reported the peroxidative effect of D-galactose in mice. As a reducing sugar, it is capable of reacting with macromolecules such as proteins, DNA and lipids without any enzymatic intervention. Due to accumulation of AGE's formed during Maillard reaction initiation of reactive oxygen species that lead to formation of lipid peroxides [24]. Increase in rat of glycation reaction by galactose may be the cause for lipid peroxidation [25]. Increase in total lipid peroxidation may be due to polyunsaturated fatty acids present in testis and epididymis [26]. Accumulation of AGE in tissue further lead to generation of ROS which ultimately resulted in increased level of TBARS in the form of MDA.

On the other hand mitochondrial membrane and DNA, also suffer the attack by ROS, due to which the mitochondrial structures get disorganized. The oxidative damage to mitochondrial DNA is well known to occur in all aerobic cells rich in mitochondria. The presence of high level of NADPH oxidase [27] and glucose-6-phosphate dehydrogenase activity [28] leads to NADPH synthesis in spermatozoa [29] in close proximity to sperm mitochondria, this lead to production of super oxide anion and H_2O_2 [30]. All these events results in the production of highly toxic oxygen free radicals- hydroxyl radicals [31].

D-galactose treated mice showed highly significant increase in the mitochondrial peroxidation in both organs, which leads to change and disorganized mitochondria. Such mitochondria as well as cellular products in the cells find their way to lysosomes. The excessive generation of ROS increases production of lipid peroxides making the membrane to lose their fluidity and integrity [32, 33].

Ethanolic extract of *Withania somnifera* along with D-galactose reduce the level of lipid peroxidation compared with D-galactose treated group of mice. Withania is traditionally used to increase vital fluids, much fat, blood, lymph and cell production. It helps to counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, rejuvenating the reproductive organs [34]. It neutralizes the free radical formation to reduce MDA formation, as it contains many flavonoids and amino acids. It also contains active ingredients viz. withanoloids [20].

Reproductive aging is associated with regressed reproductive organs like testes. Seminiferous tubules showed altered appearance and an arrest of germ cell differentiation [35]. The similar results were observed in the present work of D-galactose administered group. In testis, structural deformities were observed along with decrease in number of sperms in the lumen. The Leydig cells are found disrupted and spread all over intercellular spaces. Many investigators described about persistence of Leydig cells in aged testis by transforming into dedifferentiated Mesenchymal elements. Instead, light and electron microscopic observation of testis, it was revealed that Leydig cells were undergone degeneration and dissolution [36]. As PUFA are rich in testis and epididymis oxidation of lipid takes place faster by producing toxic reactions, in spermatozoa [37], thus for production of functional spermatozoa, protection should be provided in the form of antioxidants. The group of mice received leaf extract of *Withania somnifera* in the present investigation shows protective reappearance of structural deformities took place in earlier group received D-galactose only.

CONCLUSION

Thus from above discussion Withania may be having antioxidant property; protecting male reproductive organs from ROS and can avoid infertility. The overall findings may be helpful to the population not only to treat infertility but also to maintain normal sexual life.

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