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EFFICACY OF ASHWAGANDHA (*WITHANIA SOMNIFERA* L.) ROOT EXTRACTS IN PREVENTING STRESS INDUCED TESTICULAR DAMAGE IN RAT

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ABSTRACT
The aim of the study was to investigate whether or not chloroform and alcoholic extracts of Ashwagandha root, prevent stress induced impairment in spermatogenesis, steroidogenesis and oxidative damage in the testis by suppressing the activation of hypothalamus-pituitary- adrenal axis. Exposure of adult male rats to chronic stress (restraint,1 h followed by forced swimming exercise,15 minutes) for 1 month resulted in a significant increase in serum corticosterone concentration coupled with a significant decrease in serum testosterone concentration and a significant reduction in counts of germ cells in stage VII of spermatogenesis and epididymal sperm count. In addition, there was a decrease in the activity of anti-oxidant enzymes and concentrations of non-enzymatic anti-oxidants (ascorbic acid and tocopherol) accompanied by an increase in malondialdehyde concentration in the testis indicating oxidative damage. However, oral administration of ethanolic or chloroform extracts of *Withania somnifera* (each 10 mg/kg body weight/rat/day for 1month) or mifepristone a synthetic anti-glucocorticoid drug (10 mg/kg body weight/rat/day, last five days of the experiment) to rats 1 hour prior to stress regime did not result in changes mentioned above. The results indicate that stress alters testicular activity by suppressing the testicular steroidogenic activity and causing oxidative damage, which are effectively prevented by root extracts of Ashwagandha by suppressing stress induced activation of the hypothalamus-pituitary-adrenal axis. Therefore, crude extracts of Ashwagandha have the potential to be used for reproductive abnormalities caused by stress.

KEYWORDS: *Withania somnifera*, spermatogenesis, steroidogenesis, oxidative damage, hypothalamus-pituitary-adrenal axis.

INTRODUCTION
Urbanization and mechanization have resulted in greater exposure of humans to pollution, high consumption of salt and fat containing food, low physical activity and stress.[1] Stress leads to activation of hypothamus-pituitary-adrenal (HPA) axis, which in turn suppresses hypothamus-pituitary-gonadal (HPG) axis. Major effect of activation of hypothalamus-pituitary-adrenal (HPA) axis is the increased secretion of glucocorticoids, which on one hand impairs the testicular testosterone synthesis.[2-4] and on the other hand increases oxidative stress.[5-7] Several studies have found the apoptotic loss of germ cells.[3, 5, 8-10] and impaired testosterone secretion.[11-15] due to stress. In our earlier study [16], chronic stress has shown reduction in counts of stem spermatogonia, spermatocytes, spermatids and epididymal spermatozoa. These changes were accompanied by increased oxidative damage indicated by increased lipid peroxidation and reduced antioxidant status in the testis. The results indicated that reduced sperm count was because of loss of stem spermatogonia and subsequent stages of spermatogenic cells, which was due to oxidative damage and deficient steroidogenic activity.[16] Hence, any attempt to prevent stress induced degenerative changes in the male reproductive system has to address the problem by simultaneously suppressing testicular oxidative damage and impairment in testicular steroidogenesis. In addition, such studies have to demonstrate prevention of loss of spermatogonia due to stress and subsequent maintenance of normal sperm count under stressful conditions, as once stem spermatogonia are degenerated their number cannot be restored.[17] Since, stress cannot be avoided in the modern fast-paced society, there is an absolute need to prevent stress induced degenerative changes in the testicular activities. Herbal extracts which have been used in traditional medical systems can be a potential source of anti-stress compounds. Therefore, investigations on such herbs are needed.

The herb, Ashwagandha, *Withania somnifera* has been documented in the ancient Indian system of medicine (Ayurveda) for its ability to improve endurance against stress, general resistance against infections, retardation of the aging process and improvement of male sexual health in disorders such as psychological impotence and unexplained infertility.[18-20] For instance, Archana and Namasivayam.[21] reported that cold swimming stress

induced increase in the plasma corticosterone level, phagocytic index and avidity index in rats were reduced with subsequent administration of an aqueous extract of Ashwagandha root (100mg/kg body weight, orally for 7 days). Bhattacharya et al.[22] reported significant reduction in anxiety in rats following oral administration of 20 or 50 mg/kg body weight, withanolide glycosides. Similarly, aqueous ethanol (1:1) extract of *W. somnifera* (25 or 50 mg/kg po) for 21 days attenuated the chronic stress induced hyperglycemia, glucose intolerance, increase in plasma corticosteroid levels, gastric ulceration, male sexual dysfunction, cognitive deficits, immunosuppression and mental depression in rats. Animal trials have shown that a withanolide-free hydrosoluble fraction of *W. Somnifera* reduces the stress response induced both chemically and physically.[23] Chronic stress induced suppression of sexual activity were prevented in mice treated with Ashwagandha powder (25 or 50mg/kg body weight) for 21 days.[24] A significant elevation in the level of free radical products, i.e. TBARS and conjugated Dienes along with significant diminution in the activities of CAT, SOD and GST in the testis, prostate and seminal vesicle were found after forced swimming for 8 h/day for 28 days in rats, which were prevented by co-administration of methanolic extract of *W. somnifera* roots, *Ocimum sanctum* leaf and *Zingiber officinale* rhizome in composite manner at the concentration of 40mg/100g bw/day.[25] Patil et al.[1] showed attenuation of D- galactose induced decrease in sperm count, lipid peroxidation and mitochondrial peroxidation in the testis following administration of 2% ethanolic extract of Ashwagandha leaves for 15 days in mice. Likewise, human studies also reveal anti-stress effects of Ashwagandha. A significant increase in the seminal parameters such as motility, anti-oxidation status, sperm concentration and seminal volume and levels of vitamin C and fructose.[18-20] and increase in seminal lactate, citrate, glycerophosphocholine, motility and counts of spermatozoa.[26] were observed in infertile men following administration of 5 g of *W. Somnifera* root powder. Administration of root and leaf extract (125 or 250 mg/kg body weight) of *W. somnifera* for 60 days.[27] and 300 mg b.i.d. standardized to 1.5% with anolides, prepared from Ashwagandha root for 12 weeks.[28] resulted in significant reduction in anxiety and its comorbidities (forgetfulness, lack of sleep, etc.) in chronically stressed humans. Chandrasekhar et al.[29] reported that the ashwagandha root powder (300 mg capsules for 15, 30 and 60 days) significantly reduced the stress and anxiety in persons with chronic mental stress as assessed by the perceived stress scale.

However, none of the studies conducted earlier on prevention of stress induced alterations in testicular activities have focused on simultaneous prevention of loss of spermatogonia and oxidative damage. In addition, there are no investigations as to whether the anti-stress effects of Ashwagandha or any other herb are comparable to that of a glucocorticoid antagonist. There is a need for studies on these lines as stress effects are mainly due to actions of elevated levels of glucocorticoid. Hence, the objective of the present study was to investigate whether or not lower doses of chloroform and alcoholic extracts, compared to earlier studies prevent the stress induced impairment in spermatogenesis and steroidogenesis and oxidative damage by suppressing the activation of the HPA axis.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 200–220 g were obtained from the inbred colony of the central animal facility of the University of Mysore. The rats were maintained (3 or 2 rats/cage) under 12:12 h light and dark cycle and were provided with standard rat chow and water *ad libitum.*

Stressors

Two types of stressors were used to induce stress as described by of Grissom et al.[30]

(i) Restraint (RS): a rat was placed in an open-ended cylindrical restrainer measuring 6.7 cm in diameter and 22.2 cm in length and kept in a clean cage with bedding material for 1 h.

(ii) Forced swimming exercise (FS): rats were forced to swim in a glass chromatography jar (18 in. height×8.75 in. outer diameter), filled two thirds full of water for 15 min at room temperature.

Identification and Preparation of plant extract

Withania somnifera were collected from Mysore Ayurvedic Medical College Park, Mysore, Karnataka and were authenticated by Botanist. The herbarium number is **2063**, Departmet of Botany, University of Mysore, Mysore, India. The roots of Ashwagandha were a shade dried and a coarse powder was prepared. The powder was subjected to successive extractions using solvents with increasing polarity viz. petroleum ether, benzene, chloroform, ethanol, cold water and hot water and 0.2N sodium hydroxide using soxhlet apparatus. The ethanolic and chloroform extracts of Ashwagandha root were found to have most potent antioxidant activity in our earlier *in vitro* study.[31] Hence, in the present *in vivo* experiment these two extracts were used.

Preparation of mifepristone

Mifepristone was dissolved in 1% carboxy methyl cellulose (CMC) so as to get a concentration of 10mg/ml.

Experimental protocol

Adult male rats were randomly segregated into 6 groups (5 rats/ group). The rats in the 1st group served as controls and were intubated with 0.5ml distilled water/day/rat. The rats in the 2nd group were served as positive or vehicle control group wherein each rat was administered with 0.5ml of 1% carboxy methyl cellulose (CMC). These two groups of rats were maintained without any disturbance throughout the experimental period. The rats in 3rd group were exposed to stress regime, i.e. restraint followed by forced swimming

exercise after a gap of 4 hours daily for 1 month. Each rat in 4th group was exposed to stress regime similar to that in 3rd group and was intubated with the mifepristone, a glucocorticoid antagonist, (10mg/kg bw/0.5 ml/rat) 1 hour prior to stress regime, during the last 5 days of the experiment. The rats in 5th and 6th groups were exposed to stressors similar to those in group 3 and crude ethanolic extract (10mg/kg body weight/0.5ml/rat) and crude chloroform extract (10mg/kg body weight/0.5ml/rat) were orally administered (intubation) 1 hour prior to stress regime every day for 1 month.

Body weight of each rat was recorded at weekly intervals. At autopsy, blood samples, adrenal glands and testis were collected for biochemical analyses. The right testis of each rat were stored in −20ºC until biochemical analyses were conducted and the left testis were processed for histological studies. The epidydimis were used for determining total sperm and abnormal sperm counts.

Total sperm count

The cauda epididymis of one side of each rat was minced in 1 ml phosphate buffer saline (PBS) (pH 7.2) to obtain suspension. The a suspension was filtered through muslin cloth; an aliquot of this solution was mixed with a drop of 1% aqueous eosin and kept for 30 min for the staining of the spermatozoa. The stained filtrate was taken in a WBC pipette up to the 0.5 mark and diluted further up to the mark 11 with PBS, and mixed well and charged into Neubauer's counting chamber. The spermatozoa present in eight outer squares of 1 mm^2 area was counted. The aggregate of counts of eight squares was multiplied by 5×10^4 factor to obtain the total sperm count/ epidydimis.[32]

Abnormal sperm count

A drop of above mentioned stained spermatozoa preparation was put on a clean glass slide and a uniform smear was obtained. One thousand spermatozoa per epidydimis were observed under 400X in randomly selected areas of smear and number of spermatozoa showing head shape and tail abnormalities viz. amorphous head, pin head, bent mid-piece, curved tail, hook less head, double head was counted. The sum of counts of different abnormalities was expressed as total abnormal sperm count/1000 spermatozoa/epididymis.[32]

Spermatogenesis

The left testis were fixed in Bouin's fluid for 18 hours and dehydrated with 70% ethanol, embedded in paraffin wax and 5µm thick sections were cut and were stained with hematoxylin and eosin. The number of each category of germ cells in stage VII of the seminiferous epithelium cycle, i.e., type A spermatogonia, preleptotene spermatocytes, midpachytene spermatocytes, round spermatids and elongated spermatids was counted in ten round tubular cross sections of each rat testis. All the counts of the germ cells were converted to true counts by the formula, true counts = (crude count × section thickness)/(section thickness + nuclear diameter of germ cells).[33, 34]

Biochemical analyses

Activities of antioxidant enzymes, i.e. superoxide dismutase (SOD)[35], glutathione peroxidase (GPx)[36], catalase (CAT)[37], glutathione reductase (GR).[38] and glutathione S transferase (GST)[39] were determined in the right testis of each rat. The testicular concentrations of non-enzymatic antioxidants, i.e. ascorbic acid[40] and tocopherol[41] and a product of lipid peroxidation, malondialdehyde (MDA)[42] were determined. Further, activity of the key steroidogenic enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSDH)[43] was determined in the adrenal gland and the testis.

Serum testosterone and corticosterone concentrations

The serum concentrations of testosterone and corticosterone were estimated by ELISA using the DRG diagnostic kit manufactured by the DRG instruments Gmb H, Germany and Neogen, Germany respectively.

Statistical analysis

The mean values of each parameter were expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Duncan's multiple range test were used to determine the significant difference between mean values of different groups, fixing the minimum level of significance at $P<0.05$.

RESULTS

Body and relative adrenal and testis weight

Except the stress group, all other groups showed gains in body weight (Table 1) compared to controls. The relative weight of the adrenal gland was significantly higher in stressed rats compared to controls (Table.1) whereas there was no significant difference in the relative weight of the adrenal gland of stressed rats treated with mifepristone and crude ethanolic or chloroform extract of Ashwagandha compared to either stress group or a control group (Table1). There was a significant decrease in the relative weight of the testes in stressed and stressed rats treated with mifepristone compared to controls, whereas there was no significant difference in the relative weight of the testes of stressed rats treated with crude ethanolic or chloroform extract of Ashwagandha compared to control and vehicle control groups (Table. 1).

Table 1: **Effect of ashwagandha root extracts on stress induced alterations in body weight and weight of the adrenal gland and the testes of rat.**

Groups & Treatment	% change in body weight (g)	Relative weight	
		Adrenal gland (mg/ 100 g body weight)	Testis (g/100g body weight)
Controls	17.76±2.45[a]	10.21±1.2[a]	1.32±0.05[a]
Vehicle controls	7.27±2.27[b]	10.97±1.7[a]	1.19±0.04[a]
Stress group	-3.33±1.14[c]	21.28±2.2[b]	0.92±0.07[b]
Stress + 10mg/kg bw mifepristone	1.95±0.67[bc]	13.81±2.4[ab]	1.01±0.01[b]
Stress+ 10mg/ kg bw ethanolic extract of ashwagandha root	1.39±1.79[bc]	15.27±1.2[ab]	1.24±0.04[a]
Stress + 10mg/kg bw chloroform extract of ashwagandha root	5.40±1.54[b]	14.21±2.4[ab]	1.22±0.05[a]
F- Value df= 5, 29	**14.214 P< 0.05**	**2.46 P< 0.05**	**10.599 P< 0.05**

Values are mean ± SEM (n=5), Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different.

Adrenal and testicular 3β-HSDH activities

The adrenal 3β- HSDH activity was significantly higher in stressed rats compared to controls, vehicle controls, mifepristone and Ashwagandha extracts treated rats (Table.2). There was a significant decrease in the testicular 3β- HSDH activity in stressed rats compared to controls, vehicle controls and stressed rat treated with mifepristone and crude Ashwagandha extracts. Though it was significantly higher in mifepristone and Ashwagandha extracts treated rats than stressed rats, it was significantly lower than controls (Table 3).

Table 2: **Effect of Ashwagandha root extracts on stress induced alterations in the adrenal 3β-hydroxy steroid dehydrogenase (3β-HSDH) activity and serum corticosterone concentration in rat.**

Groups & Treatment	Adrenal 3β HSDH activity (nmol/mg/min)	Serum corticosterone concentration (ng/ml)
Controls	0.110±0.01[a]	0.64±0.22[a]
Vehicle controls	0.098±0.001[a]	0.79±0.23[ab]
Stress group	0.26±0.001[b]	3.37±0.30[c]
Stress + 10mg/kg bw mifepristone	0.082±0.002[a]	1.40±0.11[b]
Stress+ 10mg/ kg bw ethanolic extract of ashwagandha root	0.097±0.010[a]	1.07±0.03[ab]
Stress + 10mg/kg bw chloroform extract of ashwagandha root	0.121±0.012[a]	1.20±0.21[ab]
F- Value df= 5, 29	**16.43 P < 0.05**	**27.35 P< 0.05**

Values are mean ± SEM (n=5), Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by Duncan's multiple test.

Serum corticosterone levels

The serum corticosterone concentration of stressed rats was significantly higher than controls and Ashwagandha extract treated stressed rats, whereas that of controls and crude Ashwagandha extracts treated stressed rats did not significantly differ (Table 2). Though the serum corticosterone level of mifepristone treated rats was significantly lower than stressed rats, it was significantly higher than controls (Table 2).

Serum testosterone levels

There was a significant decrease in the serum testosterone concentration in stressed rats compared to controls, vehicle treated controls and chloroform extract of Ashwagandha treated rats, whereas that of mifepristone and ethanolic extract of Ashwagandha treated rats though showed significant increase over stressed rats, it was significantly lower than controls (Table 3).

Table 3: Effect of Ashwagandha root extracts on stress induced alterations in testicular 3β-hydroxy steroid dehydrogenase (3β HSDH) activity and the serum concentration of testosterone in rat.

Groups & Treatment	Testicular 3β HSDH activity (nmol/mg/min)	Serum testosterone concentration (ng/ml)
Controls	0.47±0.03[a]	1.42±0.06[a]
Vehicle controls	0.43±0.01[ab]	1.37±0.13[a]
Stress group	0.21±0.02[d]	0.55±0.04[c]
Stress + 10mg/kg bw mifepristone	0.31±0.02[c]	1.03±0.07[b]
Stress+ 10mg/ kg bw ethanolic extract of ashwagandha root	0.35±0.03[c]	1.17±0.05[b]
Stress + 10mg/kg bw chloroform extract of ashwagandha root	0.39±0.03[bc]	1.43±0.07[a]
F- Value df= 5, 29	**17.54 P < 0.05**	**18.38 P<0.05**

Values are mean ± SEM (n=5), Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different.

Total and abnormal sperm count

Total sperm count was significantly decreased in the stressed rats compared to all other groups. Though sperm count in the Ashwagandha extracts and mifepristone treated stressed rats was significantly higher than stressed rats, it was significantly lower than controls (Table 4).

Abnormal sperm count was significantly increased in stressed rats compared to controls, vehicle controls and stressed rats treated with mifepristone and Ashwagandha extracts (Table 4).

Table 4: Effects of Ashwagandha root extracts on stress induced alterations in epididymal total and abnormal sperm count in rat.

Groups & Treatment	Total Sperm count (millions/epidydimis)	Number of abnormal spermatozoa /1000 spermatozoa
Controls	144.51±5.57[a]	68.20±8.02[a]
Vehicle controls	133.06±2.77[ab]	76.10±5.77[a]
Stress group	63.76±2.63[d]	126.80±9.11[b]
Stress + 10mg/kg bw mifepristone	92.64±3.58[c]	103.00±11.79[ab]
Stress+ 10mg/ kg bw ethanolic extract of ashwagandha root	111.48±7.93[b]	84.00±12.08[a]
Stress + 10mg/kg bw chloroform extract of ashwagandha root	121.71±7.78[b]	78.23±9.34[a]
F- Value df= 5, 29	**25.98 P<0.05**	**3.39 P<0.05**

Values are mean ± SEM (n=5), Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different.

Histomorphology of the testis

The testis of controls and vehicle treated controls and rats treated with Ashwagandha extracts revealed the normal structure and seminiferous tubules were replete with germ cells at different stages of spermatogenesis and abundant spermatozoa (Fig. 1 a, b, e and f) whereas the seminiferous tubules of stress rats and stressed rats treated with mifepristone (Fig.1 c and d) were shrunken with vacuolization in the seminiferous epithelium and contained fewer spermatozoa compared to controls, vehicle controls and stressed rats treated with Ashwagandha extracts.

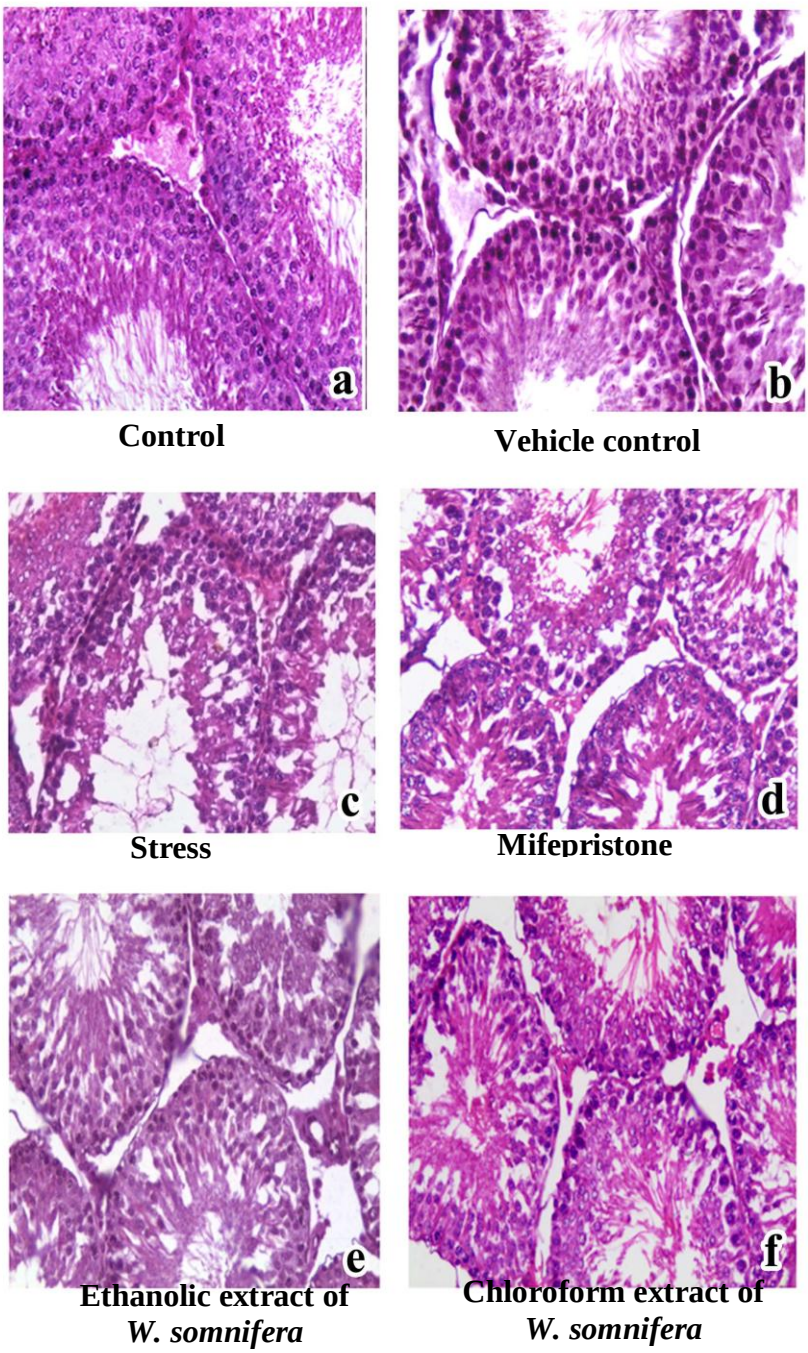

Fig. 1. a, b, c, d, e & f: Photomicrographs of the cross sections of the testis of control (a), vehicle control (b), stressed rat (c), stressed rat treated with mifepristone (d), stressed rat treated with ethanolic (e) and chloroform (f) extracts of Ashwagandha root. (H & E, 200X).

Differential counts of stage 7 of spermatogenesis

Counts of A spermatogonia, preleptotene and mid pachytene spermatocytes, round and elongated spermatids of stage 7 of spermatogenesis were significantly reduced in the stressed and mifepristone treated stressed rats compared to controls, vehicle controls and Ashwagandha extracts treated rats. Though these counts were lower in Ashwagandha extract treated stressed rats compared to controls, they did not differ from vehicle treated controls (Table 5).

Table 5: Effects of Ashwagandha root extracts on stress induced alterations in counts of different categories of germ cells in stage VII of spermatogenesis in rat.

Groups & Treatment	Mean number ± SEM/tubule cross section				
	Type A spermatogonia	Preleptotene spermatocytes	Midpachytene spermatocytes	Round spermatids	Elongated spermatids
Controls	3.40±0.15^{a}	17.15±0.33^{a}	35.91±1.35^{ab}	112.34±2.96^{ab}	138.00±2.64^{a}
Vehicle controls	2.74±0.19^{b}	15.98±0.18^{b}	33.89±1.56^{b}	108.25±1.35^{bc}	128.60±2.42^{ab}
Stress group	2.11±0.09^{c}	13.73±0.36^{c}	29.58±0.68^{c}	98.98±1.54^{d}	103.60±3.94^{d}
Stress + 10mg/kg bw mifepristone	2.12±0.08^{c}	14.35±0.62^{c}	29.68±1.04^{c}	100.99±1.66^{cd}	112.00±3.75^{cd}
Stress+ 10mg/ kg bw ethanolic extract of ashwagandha root	2.64±0.16^{b}	15.90±0.9^{b}	37.98±1.77^{a}	110.97±3.06^{ab}	120.20±4.02^{bc}
Stress + 10mg/kg bw chloroform extract of ashwagandha root	2.85±0.22^{b}	17.24±0.17^{a}	38.45±1.43^{a}	117.15±4.05^{a}	124.40±3.8^{b}
F- Value df= 5, 29	**9.59 P<0.05**	**17.67 P<0.05**	**10.12 P<0.05**	**6.99 P<0.05**	**12.15 P<0.05**

Values are mean ± SEM (n=5), Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different.

Activities of testicular antioxidant enzymes

The testicular CAT activity was significantly reduced in stressed rats compared to all other groups, whereas that in vehicle controls, mifepristone and Ashwagandha extracts treated stressed rats was significantly lower than controls (Table 6). The testicular SOD activity was significantly reduced in stressed rats compared to controls, vehicle controls and Ashwagandha extracts treated stressed rats, whereas that in mifepristone treated stressed rats though showed an increase it was significantly lower than controls (Table 6).

The activities of GPx, GST (Table.6) were significantly reduced in stressed rats compared to controls, vehicle controls and mifepristone and Ashwagandha extracts treated stressed rats, whereas GR activity was significantly reduced in stressed rats compared to all other groups but it was significantly lower in mifepristone and Ashwagandha extracts treated stressed rats compared to controls and did not differ from vehicle treated controls (Table 6).

Table 6: Effects of Ashwagandha root extracts on stress induced alterations in the activities of testicular catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S transeferase (GST) and glutathione reductase (GR) of rat.

Groups & Treatment	CAT (nmol/mg/min)	SOD (U/mg protein)	GPx (µmol/mg/min)	GST (µmol/mg/min)	GR (U/ml)
Controls	0.0052±0.0002^{a}	2.69±0.03^{a}	0.0088±0.0001^{a}	31.27±1.89^{a}	21.69±0.19^{a}
Vehicle controls	0.0043±0.0002^{bc}	2.39±0.030^{ab}	0.0075±0.0001^{a}	32.36±1.35^{a}	20.15±0.29^{ab}
Stress group	0.0015±0.0001^{d}	1.77±0.06^{c}	0.0021±0.0004^{b}	15.33±0.30^{b}	12.90±016^{d}
Stress + 10mg/kg bw mifepristone	0.004±0.0001^{c}	2.01±0.03^{bc}	0.0071±0.0003^{a}	30.46±1.05^{a}	18.48±0.46^{c}
Stress+ 10mg/ kg bw ethanolic extract of ashwagandha root	0.0047±0.0001^{b}	2.29±0.04^{ab}	0.0074±0.0006^{a}	33.50±1.79^{a}	19.26±0.28^{bc}
Stress + 10mg/kg bw chloroform extract of ashwagandha root	0.0046±0.0002^{b}	2.36±0.04^{ab}	0.0076±0.0009^{a}	34.04±1.44^{a}	20.44±0.48^{b}
F- Value df= 5, 29	**107.69 P < 0.05**	**4.27 P < 0.05**	**9.73 P < 0.05**	**9.78 P < 0.05**	**44.97 P < 0.05**

Values are mean ± SEM (n=5), Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different, CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidise; GST, glutathione-S-transferase; GR, glutathione reductase.

Concentrations of ascorbic acid and tocopherol

The concentrations of testicular ascorbic acid and tocopherol were significantly reduced in stressed rats compared to controls, vehicle controls and mifepristone and Ashwagandha extracts treated stressed rats. These parameters of mifepristone treated stressed rats were significantly lower than controls and higher than stressed rats (Table.7).

Table 7: Effects of Ashwagandha root extracts on stress induced alterations in the concentrations of ascorbic acid, tocopheroland and malondealdehyde (MDA) in the testis of rat.

Groups & Treatment	(µmol/mg protein)		MDA(nmol/g protein)
	Ascorbic acid	tocopherol	
Controls	2.69±0.17[a]	2.61±0.19[a]	8.77±0.97[a]
Vehicle controls	2.53±0.13[a]	2.71±0.08[a]	9.79±0.64[a]
Stress group	1.31±0.03[c]	1.53±0.12[c]	16.13±1.35[b]
Stress + 10mg/kg bw mifepristone	2.08±0.19[b]	2.27±0.06[b]	7.78±2.14[a]
Stress+ 10mg/ kg bw ethanolic extract of ashwagandha root	2.35±0.09[ab]	2.66±0.10[a]	8.21±1.16[a]
Stress + 10mg/kg bw chloroform extract of ashwagandha root	2.57±0.11[a]	2.72±0.10[a]	8.58±0.85[a]
F- Value df= 5, 29	**14.82 P < 0.05**	**16.09 P < 0.05**	**7.91 P<0.001**

Values are mean ± SEM (n=5), Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different.

Malondialdehyde concentration

The concentration of MDA in the testis was significantly higher in stressed rats compared to controls, vehicle controls and mifepristone and Ashwagandha extracts treated stressed rats (Table 7).

DISCUSSION

In the present day fast paced society, human beings are exposed to a variety of stressors and undergo incidents of chronic stress. Hence, there is a need to prevent the negative effects of stress despite undergoing stressful experiences. Stress adversely affects the reproductive system. The action is mediated by the activation of hypothalamic-pituitary-adrenal axis that exerts intense inhibitory effects on the hypothalamic-pituitary-gonadal axis subsequently leading to reproductive failure in both males and females.[44, 45] Hence, there is a need to prevent the negative effects of stress despite undergoing stressful experiences. The present study reveals the possibility of prevention of stress induced alterations in spermatogenesis, steroidogenesis and testicular antioxidant status.

Since the activation of HPA axis and consequent activation of the adrenal cortex and increase in corticosterone secretion is a familiar stress response seen in vertebrates[45-47], an increase in activity of the adrenal 3β-HSDH and serum corticosterone levels concomitant with an increase in the adrenal gland weight in rats exposed to restraint and forced swimming daily for one month in the present study indicate activation of HPA axis leading to an increase in adrenocortical activity. It is to be noted that despite every day exposure, the adrenal gland responded to stressors, thereby indicating a state of chronic stress in rats. Further, in this study, an increase in serum corticosterone levels was accompanied by a significant decrease in the activity of the key testicular steroidogenic enzyme, 3β-HSDH and serum testosterone levels concomitant with reduction in the weight of testes, counts of germ cells including androgen sensitive germ cells in stage VII of spermatogenic cycle, total sperm count and antioxidant enzyme activities and an increase in testicular MDA concentration and abnormal sperm count. These alterations in the testes of chronically stressed rats are indicative of impairment in testicular steroidogenic and spermatogenic activities coupled with oxidative damage.

Impairment in spermatogenesis might be due to stress induced deficiency of hormones regulating the spermatogenesis on one hand and apoptotic loss of germ cells on the other. Indeed earlier studies have reveled suppression of testosterone biosynthesis stress due to reduction in gonadotrophin levels[48], gonadotrophin sensitivity of the testis[3, 48] and apoptotic loss of the Leydig cells[49, 50] due to stress induced hypersecretion of glucocorticoids. In addition stress induced increased glucocorticoids cause apoptosis of spermatogonia and other spermatogenic cell types.[3, 5] Thus apoptotic loss of germ cells and reduced hormone secretion might reduce the spermatogenic out put ultimately resulting in the decrease in epididymal sperm count.

Further, chronic stress also known to increase the oxidative damage which may in turn interfere with testicular activity.[16] The lipid peroxidation, a major consequence of oxidative stress biomarker causes profound changes in membrane structure that might lead to cell death.[51] Spermatozoa are particularly susceptible to oxidative damage due to their unique structural composition[52] and lipid peroxidation induces loss of membrane integrity.[53, 54] Further testicular tissues are rich in polyunsaturated fatty acid content and posses poor antioxidant defense and hence more prone to oxidative damage.[55, 56] It is known that hypersecretion of glucocorticoids under stress causes oxidative damage.[5, 6] Thus hormonal imbalance and oxidative damage under stress contribute to reduced sperm output. Thus far a single study has not focused on both of these factors in altering testicular activities in a given stress regime. This approach is essential because remedial measures to prevent stress effects must be able to prevent

both. The present study provides an experimental evidence for this view. Chronic stress due to every day exposure to stressors in the present study resulted in activation of HPA axis as shown by increased corticosterone levels and adrenal 3β-HSDH activity, which was accompanied by suppression of spermatogenic and steroidogenic activity as indicated by decrease in the counts of spermatids and other germ cells in stage VII of spermatogenesis, epididymal sperm counts, reduction in testicular 3β–HSDH activity and serum testosterone levels. The suppression of spermatogenic activity might be due to glucocorticoid induced deficiency of testosterone, as stage VII of spermatogenesis in rat is known to be highly androgen sensitive.[57] These testicular changes in chronically stressed rats were accompanied by compromise in antioxidant defense system, as shown by reduction in activities of antioxidant enzymes (CAT, SOD, GPx, GR and GST) and concentration of non-enzymatic antioxidants (ascorbic acid and tocopherol) coupled with increased lipid peroxidation in the testis. It is logical, based on above discussion that any treatment to prevent reproductive effects of stress, must be able to prevent stress induced reproductive hormone imbalance as well as testicular oxidative damage to maintain normal sperm count despite undergoing stress. Since adverse effects of stress on the testis i.e hormonal imbalance.[3, 58] and oxidative damage.[5, 6] are manifestations of hyper secretion of glucocorticoids, suppression of stress induced increase in adrenocortical activity might result in normal testicular activity despite animal undergoing stressful experiences. The likely candidates to prevent activation of HPA axis under stress would be antagonists of either glucocorticoids or CRH. Mifepristone (RU-486), a synthetic steroid antagonizes the action of glucocorticoids comparatively at the receptor level[59] by binding to glucocorticoid receptors (GR II). In the present study mifepristone has been used as glucocorticoid antagonist to find out whether it prevents stress induced testicular alterations by suppressing HPA axis. Indeed, the mifepristone treated stressed rats showed higher testicular 3β-HSDH activity and testosterone levels coupled with an increase in antioxidant enzyme activities and a decrease in MDA levels compared to stressed rats, thereby indicating intervention of stress effects. The results suggest that the glucocorticoid antagonist could intervene with stress effects on spermatogenesis as well as antioxidant status. However, mifepristone treatment is not advisable for long term administration as it has several undesirable effects.[60, 61] Therefore in the present study mifepristone was administrated in the last 5 (10 mg/kg bw) days of the experimental period as the previous reports says that prolonged usage of this drug leads to adverse effects[60, 61] and the treatment was used only to demonstrate the possibility of prevention of testicular alteration despite animal undergoing stress. There is a need for alternative, and one of the choices could be naturally occurring molecules in herbs used either as food or medicine and are in usage for decades without undesirable effects. One of such herbs is the Ashwagandha, *Withania somnifera* which is used in the Ayurveda, an Indian system of medicine. It is known to suppress cold stress induced [21] and footshock induced corticosterone levels[62] in rats. In addition *W. somnifera* root extracts treatment improved seminal parameters viz. sperm motility, antioxidant status, sperm count and volume of semen[18, 20, 26] as well as increased testosterone levels[19] in infertile males. Though these studies demonstrate suppression of stress induced glucocorticoids levels and reproductive effects, very high doses of root extracts have been used. For instance, in rats 100 mg/kg body weight[21], and 50 mg/kg body weight[22] and 5 g in human[18-20, 26] were administered. In addition, thus far there are no reports whether treatment by Ashwagandha or any other herb could prevent testicular alterations in stressed animals as these studies have mainly focused on seminal and blood parameters. It is obvious that prevention of testicular alteration is essential as it is the source of seminal spermatozoa. Present study focused on testicular changes and a relatively lower dose of alcoholic and chloroform extracts of Ashwagandha root i.e 10 mg/kg body weight were administered. In stressed rats treated with Ashwagandha extracts, the serum corticosterone level, adrenal 3β HSDH activity, abnormal sperm count, concentration of melondealdehyde were significantly decreased compared to stressed rat whereas concentration of serum testosterone, total epididymal sperm count, counts of different germ cells in stage VII of spermatogenesis, activities of antioxidant enzymes (SOD, CAT, GPx, GST and GR) were significantly increased in contrast to stressed rats. It is evident from the results that Ashwagandha root extracts at dose levels lower than earlier studies prevent stress induced alterations in gametogenic activity as well as antioxidant status. That, neither the testicular histomorphology and steroidogenic activity nor counts of different categories of germ cells in Ashwagandha root extracts received stressed rats did not markedly vary from controls reveal that normal sperm counts in these rats is due to optimal testicular function. It is to be noted that hormonal imbalance as well as diminished antioxidant status due to stress were simultaneously prevented by Ashwagandha resulting in near normal gametogenic activity in rats despite undergoing stress. The fact that the adrenocortical activity (3β-HSDH activity) and serum corticosterone levels in Ashwagandha extract treated stressed rats resembled controls indicate that these extracts prevented testicular alterations by suppressing activation of HPA axis in stressed rats. It is interesting to note that, mifepristone, a potent glucocorticoid antagonist , though prevented stress induced alterations in the testis, the Ashwagandha root extracts were more potent at the given dose level as some of the parameters, viz. testis weight and histomorphology, counts of germ cells, SOD activity and concentration of non-enzymatic antioxidants were though better maintained in mifepristone treated stressed rats compared to stressed rats these did not resemble controls, whereas majority of parameters in Ashwagandha root extracts treated stressed

rats resembled controls.

Primary spermatogonial population is continuously renewed to replace germ cells that had progressed to differentiate into spermatozoa and released from the seminiferous tubules. This process ensures continuous production and maintenance of species specific sperm count. Hence, interruption in this process of renewal of spermatogonial population, might result in a decrease in epididymal sperm count. In the present study, there was a significant decrease in counts of spermatogonia in stressed rats which was accompanied by a substantial drop in epididymal sperm count. Therefore the reduced sperm count was due to loss of spermatogonia and subsequent stages of speramtogenic cells. The loss of germ cells might be due to deficiency of testosterone as well as oxidative damage in stressed rats as mentioned in above. It is known that stem spermatogonia although proliferate to generate new spermatogenic waves to replace the spermatozoa that are released, once degenerated are not newly formed in adults[17] as species specific number of spermatogonia establish the spermatogonial population during development. Hence, loss of stem spermatogonia caused by any factor leads to decrease in spermatogonial population resulting in reduced sperm output and sperm count. For instance, in our earlier study[16] it was found that in long term (more than 2 months) chronically stressed rats there was irreversible decrease in spermatogonial count which was accompanied by drop in epididymal sperm count. Hence, studies on protective effects of any herbal extracts against stress induced impairment in spermatogenesis have to demonstrate prevention of loss of spermatogonia in stressed animals and subsequent maintenance of normal sperm count. However, none of the earlier studies on protective effects of herbal extracts on stress induced testicular alterations focused on this aspect. The present study clearly demonstrates that chloroform and alcoholic extracts of Ashwagandha roots prevent loss of spermatogonia and maintain normal sperm count in stressed rat as neither number of spermatogonia nor epididymal sperm count in stressed rats did not significantly differ from controls.

Our present study gains importance due to the fact that, male infertility contributes to 10-30% of clinically infertile couples[63] and oxidative stress is a major causative factor for infertility and several factors viz. cigarette smoking, environmental pollutants, ionizing radiation, xenobiotics and stress are known to induce oxidative stress. In addition stress is also known to cause reproductive hormone imbalance as discussed above. Ashwagandha, is a time tested medicinal herb, without marked toxic effects.[64, 65]

CONCLUSION

A lower dose (10 mg/kg body weight) of alcohol or chloroform extract of roots of Ashawagandha, compared to earlier studies[18- 22, 26] not only prevents oxidative damage but also maintains near normal spermatogenesis and serum androgen levels in stressed rats. In addition, since these extracts exert their effect by suppressing stress induced hypersecertion of corticosterone, these might also prevent stress induced alterations in other physiological processes which need to be investigated in future studies.

Conflict of interests

The authors declared no conflict of interest.

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