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Delayed response of epididymal sperm characteristics and testicular oxidative stress following EGME exposure: Ameliorating potential of *Withania somnifera* root extract

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ABSTRACT

Swiss strain male mice were administered orally with ethylene glycol monomethyl ether (EGME) for 6 and 10 days and studied the epididymal sperm characteristics and testicular oxidative stress 24 hours after each duration. Withdrawal study on the same parameters was carried out 20 and 45 days after the mice received EGME for 10 consecutive days. Efficacy of *Withania somnifera* (*Ws*) root extract was also studied in the mice received EGME for 10 days only, followed by oral administration of WS, from 11th day to 30th day. Treatment with *Ws* root extract was then terminated and studied the effects on 31st day. EGME treatment for 6 and 10 consecutive days did not cause significant alterations in the percentage of motile, viable, count and abnormal spermatozoa in the epididymis as well as the oxidative stress in the testis. However significant reductions were noted in these sperm indices with a significant increase in abnormal spermatozoa in the epididymis of 20 days EGME-withdrawal group. Oxidative stress in the testis of the mice of the same group was also elevated as evidenced by significant reductions in the activities of antioxidant enzymes (SOD, catalase and GPX) and increase in lipid peroxidation. Forty five days after withdrawal of the treatment, as well as supplementation with *Ws* for 20 days in 10 days EGME-treated mice resulted in significant reinstatement in these parameters. The study suggested that the toxicity of EGME noticed during early treatment withdrawal period, may be prevented by *Ws* root extract therapy.

INTRODUCTION

The glycol ethers represent a diverse series of compounds having properties which make them widely suited for a variety of solvent applications. They are used in industries as jet fuel deicers, photography, inks and coatings, dyeing and in the manufacture of plasticizers and printed circuit boards. Among ethylene glycol ethers, ethylene glycol monomethyl ether (EGME; 2-methoxyethanol) is a widely used solvent in semiconductor, chemical and food industries. It is also used in dyeing leather. A lot of literature is available regarding toxicity of EGME in various species. It has been reported that rapidly dividing cells with high metabolism are more vulnerable to EGME-treatment (Johanson, 2000; Boatman, 2005; Bagchi and Waxman, 2008). Rapidly dividing cell systems such as bone marrow, thymus, spleen and testicular epithelium germ cells appear to be the primary target organs following exposure to EGME. Wiley *et al.* (1938) were the first to report that treatment with EGME causes damage to the testis of rabbit. This observation has been confirmed and extended by several authors (Watanabe *et al.*, 2000, Boatman, 2001; Boatman and Knaak, 2001; Multigner *et al.*, 2005). EGME is considered as the most potent toxicological alkyl ether inducing testicular damage in the rat (Watanabe *et al.*, 2000) and has noticeable adverse effects on sperm concentration and quality in the rat, mouse and rabbit (Boatman, 2001; Boatman and Knaak, 2003; Multigner *et al.*, 2005). It adversely affects the mating performance and impairs the epididymal sperm characteristics in the rat (Wang *et al.*, 2006). There are few reports suggesting that EGME-induced testicular toxicity is mediated through the inhibitor of primary flavoprotein dehydrogenases (Takei *et al.*, 2010) and oxidative stress (Bagchi and Waxman, 2008). The preventing approach for such induced

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toxicities using herbal extracts has become a major area of concern in the reproductive health. Several medicinal plants have been used in the treatment of stress-induced infertility. Medicinal plants like *Asparagus racemosus*, *Bacopa monnieri*, *Phyllanthus emblica*, *Withania somnifera*, *Tribulus terrestris* etc. are widely reported for their antioxidant properties (Chaurasia *et al.*, 2000; Staugh *et al.*, 2001; Zheleva-dimitrova *et al.*, 2012).

Protective effects of some medicinal plants have also been reported against ethylene glycol-induced toxicity in rats. *Rubia cardifolia* protects the Ethylene glycol-induced nephrotoxicity. Adepu *et al.* (2015) and Lulat *et al.* (2016) studied the antiurolithiatic effect of *Alternanthera tenella* colla and lithocare against ethylene glycol-induced urolithiasis in rats, respectively. Antioxidant properties of biflavonoid kolaviron, from the seed of *Garcinia kola* has been reported against ethylene glycol monoethyl ether (EGEE)-induced reproductive toxicity in male rats by reducing the oxidative stress and improving the quality and quantity of spermatozoa (Adedara and Farombi, 2012).

Withania somnifera (*Ws*), is one of the members of generally regarded as safe category of plants that can be used for the therapeutic purposes (Bolleddula *et al.*, 2003). It is one of the traditional medicinal plant, widely used in Ayurvedic medicine system of India. It is also known `as Ashwagandha, Indian ginseng and winter cherry. Charak Sanghita mentions the therapeutic importance of its different parts. This plant contains several biologically active chemical constituents such as alkaloids (isopellertierine, anferine), steroidal lactones (withanolides, withaferins), saponins and withanolides. Historically, this plant has been used as an adaptogen, aphrodisiac, antioxidant, liver tonic, astringent, anti-inflammatory agent and more recently to treat ulcers, venom toxins, bacterial infection, and senile dementia (Singh *et al.*, 2010). Reports of Ilayperum and co-authors (2002) have suggested the use of *Ws* for the treatment of libido, inflammation, anxiety, Parkinson's disease, cognitive and neurological disorders. Archana and co-authors (1999) have reported the adaptogenic and antistressor properties of *Ws*. It also possesses anti-oxidantive properties (Bhattacharya *et al.*, 2001; Jaleel *et al.*, 2008).

The root of *Ws* contains several alkaloids, withanolides, flavonoids and reducing sugars (Umadevi, 1996). More than 20 active constituents have been reported to date, including withaferin A, sitoindosides VII-X, withanosides I-VII, choline, and β-sitosterol (Ganzera *et al.*, 2003). The root extract of *Ws* has been shown to have anti-stress, anti-inflammatory, anti-arthiritic, anti-pyretic, analgesic, anti-oxidant and immunomodulatory properties (Dhuley, 2000; Davis and Kuttan, 2002; Prakash *et al.*, 2002; Gupta *et al.*, 2003). Ameliorating potential of *Ws* has also been reported against arsenic, in impairing fertility of the males (Kumar *et al.*, 2015). Further, *Ws* also improves galactose-induced stress in the rat (Patil *et al.*, 2012).

Due to presence of richest natural sources of bioactive compounds in the whole root of *Ws*, reputed for its antioxidant potential, its extract has been planned to be used for the proposed study. Further no studies have been reported regarding the efficacy of *Ws* on EGME-induced reproductive toxicity in the males. Thus, the present study deciphers the ameliorating potentiality of the root extract of *Withania somnifera* on the epididymal sperm characteristics and testicular oxidative stress in Ethylene Glycol Monomethyl Ether-exposed mice.

MATERIALS AND METHODS

Preparation of plant extract

The roots of *Withania somnifera* were collected from local market of Varanasi, got identified in the Department of Botany, BHU, Varanasi, India. The roots were cleaned, washed with distilled water and blotted with blotting paper. They were shade dried and crushed in electric grinder to make the coarse powder. The coarse powder was extracted with 70% ethanol (1:10) in a Soxhlet apparatus and then dried at reduced pressure. The extract was stored at 4˚C in air tight bag and dissolved in distilled water for animal treatment.

Animals

Thirty adult male mice of Swiss strain were used in the present study. The animal study protocol was approved by the Animal Ethical Committee, BHU, Varanasi (F.Sc./IAEC/2014-15/0333). The animals were maintained in well ventilated room at 12 hours light and 12 hours dark. They were fed with pelleted food and drinking water *ad libitum*. The initial body weight of all the animals was recorded.

Experimental design

The animals were divided into six groups of five each. Mice of Group I served as vehicle-treated control. EGME (600 mg/kgBW/day) was administered in Groups II and III for 6 and 10 consecutive days, respectively. Mice in Group IV and V were administered with same dose of EGME for 10 consecutive days and sacrificed 20 and 45 days after withdrawal of the treatment. Animals of Group VI were administered with EGME (600 mg/kgBW/day) for 10 days and then administered with *Ws* (100 mg/kgBW/day) only for 20 days that is from 11th to 30th day, followed by their sacrifice on 31st day. EGME and *Ws* were dissolved in double distilled water and administered through oral rout. Twenty four hours after the last treatment, final body weight of the animals was recorded and euthanized by cervical dislocation. The testis and epididymis were dissected out soon after euthanization, cleared of fat and washed with saline. The testis was stored in the deep freezer for estimation of antioxidant enzymes while the epididymis was used for the sperm assessment immediately following the sacrifice.

Epididymal sperm assessment

Cauda epididymidis was minced thoroughly in the normal saline (0.9% NaCl) at 37˚C and the suspension was then used for sperm analysis according to WHO laboratory manual (2010).

Sperm motility

The sperm motility was observed immediately under the microscope at 40X by placing a drop of prepared sperm suspension on the pre-warmed slide and covered with the pre-warmed coverslip. Spermatozoa showing any degree of movement were considered to be motile. More than one hundred spermatozoa were observed to calculate the percentage motility.

Sperm viability

The viability of spermatozoa was studied by preparing smear of a drop of suspension using supra-vital staining technique with eosin-nigrosin stain. About one hundred spermatozoa including viable and dead, were scored from different region of the smear prepared on the slide to calculate the viability percentage. Spermatozoa appearing pinkish were considered dead, while those appearing colourless as viable.

Sperm count

A twenty fold dilution was made by mixing the sperm suspension with the spermicidal solution ($NaHCO_3$: 4g + phenol: 1g in 100 ml of distilled water). A haemocytometer with improved Neubauer ruling was used for counting the spermatozoa.

Sperm morphology

For evaluation of the sperm morphology, same suspensions as used for the viability test, were examined. Evaluation of sperm abnormality was based on the criteria of Wyrobek and Bruce (1975) and Zaneveld and Polakoski (1977). About one hundred spermatozoa both normal and abnormal were counted from different fields to calculate the percentage abnormality.

Homogenate preparation

Ten percent (w/v) homogenate of the testis was prepared with the aid of 50 mM phosphate buffer (pH- 7.0), as per requirement. The whole homogenate was first centrifuged at 10,000 X g for 20 minutes at 4˚C (Vaithinathan *et al.*, 2008). The supernatant was collected and kept at −20˚C till further investigation. The supernatant was used for the enzymes assay after estimating the protein content by the method of Lowry *et al.* (1951).

Antioxidant enzymes analysis

Lipid peroxidation estimation

The concentration of malonaldehyde (MDA) was measured in the supernatants by the method of Okhawa *et al.*, (1979). The reaction mixture was prepared by adding 200 µl supernatant, 100 µl of 0.8% BHT (Butylated Hydroxy Toluene), 200 µl SDS (Sodium dodecyl sulphate), 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous solution of TBA (Thiobarbituric acid). Reaction mixture was then heated at 95˚C in water bath for 60 minutes. Then the mixture was cooled and centrifuged at 2000 rpm for 10 minutes. Absorbance of supernatant was measured in a spectrophotometer at 532 nm against blank containing all the reagents except the test sample. The values were expressed as nanomole MDA produced per milligram protein.

Superoxide dismutase

Superoxide dismutase (SOD) was assayed by the method of Murklund and Marklund (1974). The assay mixture was prepared by adding 2.4 ml of Tris – HCL (50 mM) containing 1 mM EDTA (pH 7.6), 300 µl pyrogallol (0.2 mM) and 100 µl enzyme source. The increase in absorbance was measured immediately at 420 nm, against blank containing all components except the enzyme source and pyrogallol, at 10 seconds interval for 3 minutes on spectrophotometer. The enzyme activity was expressed as unit per milligram protein.

Catalase

Catalase was assayed by the method of Claiborne (1985). The assay mixture contained 2.4 ml of phosphate buffer (50 mM, pH 7.0), 10 µl of H_2O_2 (19 mM) and 50 µl enzymes source. The decrease in absorbance was measured immediately at 240 nm, against a blank containing all the components except the enzyme source, at 10 seconds interval for 3 minutes on a spectrophotometer. The enzyme activity was expressed as micromoles H_2O_2 consumed per minute per milligram protein.

Glutathione peroxidase

Glutathione peroxidase (GPx) was assayed by the method of Flohe and Gunzler (1984). Enzyme assay was carried out by pipetting 750 µl of potassium phosphate buffer (0.1M, pH 7.0), 60 µl of NADPH (2.25 mM in 0.1% $NaHCO_3$), 15 µl of glutathione reductase (7.1 µl/ml) and 25 µl of reduced glutathione (11.52 mg/ml), 50 µl of supernatant and 100 µl of H_2O_2. The extinction of the sample was recorded at 340 nm every minute for the period of 120 seconds in a spectrophotometer. The glutathione peroxidase activity was expressed in units per milligram of protein.

Statistical analysis

The values were represented as mean ± S.E. in each group. All the data were analyzed statistically by one way ANOVA followed by Newman-Keul's test for comparison of the groups. The values for $P < 0.05$ were considered significant.

RESULTS

Body weight

No significant differences were obsorved between the initial and the final body weights of the mice in any groups (Table 1).

Table 1: Effect of ethanolic extract of *Ws* on body weight of EGME intoxicated male mice.

Groups	Initial Body Weight (g)	Final Body Weight (g)
I: Control	22.6 ± 0.4	24.8 ± 0.38
II: EGME (600 mg/kgBW/day for 6 days)	26.8 ± 0.49	27.4 ± 0.25
III: EGME (600 mg/kgBW/day for 10 days)	25.6 ± 1.17	26.4 ± 0.98
IV: EGME* (600 mg/kgBW/day for 10 days)	26.0 ± 0.63	27.2 ± 0.49
V: EGME** (600 mg/kgBW/day for 10 days)	24.8 ± 1.62	26.8 ± 0.8
VI: EGME (600 mg/kgBW/day for 10 days) + *Ws* (100 mg/kgBW/day from 11th to 30th day)	25.8 ± 0.66	27.6 ± 0.34

(Values are mean ± SEM of five animals).
* 20 days withdrawal of the treatment
** 45 days withdrawal of the treatment

Sperm assessment

No significant alterations were noticed in the motility, viability and count of epididymal spermatozoa, in the mice sacrificed 6 (Gr. II) and 10 days (Gr. III) after treatment with EGME, comparable to the control (Gr. I). Percentage of morphological abnormal spermatozoa obtained from the cauda epididymidis of the mice of Gr. II and III also remained comparable to that of the mice of Gr. I. By contrast, in the mice treated with EGME for 10 days, followed by their sacrifice 20 days after withdrawal of the treatment (Gr. IV), these sperm parameters declined significantly except that of the percentage of sperm abnormality that was elevated significantly, compared to the control. However, 45 days after withdrawal of the treatment (Gr. V) and WS supplementation from 11th to 30th day after 10 days EGME treatment withdrawal (Gr. VI) resulted in restoration in such sperm indices, similar to the control (Table 2).

Table 2: Effect of ethanolic extract of *Ws* on sperm parameters of EGME intoxicated mice.

Groups	Motility (%)	Viability (%)	Abnormality (%)	Count (x10^6)
I: Control	75.40 ± 1.03	74.8 ± 1.36	20.15 ± 1.73	17.34 ± 0.169
II: EGME (600 mg/kgBW/day for 6 days)	72.50 ± 1.04	69.25 ± 1.49	20.25 ± 0.854	17.23 ± 0.358
III: EGME (600 mg/kgBW/day for 10 days)	69.60 ± 4.58	67.4 ± 4.5	18.2 ± 0.141	16.19 ± 1.53
IV: EGME* (600 mg/kgBW/day for 10 days)	43.83 ± 4.57^a	46.2 ± 2.42^a	53.82 ± 5.03^a	5.05 ± 1.11^a
V: EGME** (600 mg/kgBW/day for 10 days)	72.00 ± 1.95^b	64.6 ± 1.89^b	20.2 ± 1.02^b	15.14 ± 2.16^b
VI: EGME (600 mg/kgBW/day for 10 days) + *Ws* (100 mg/kgBW/day from 11th to 30th day)	61.40 ± 1.23^c	62.25 ± 1.03^c	20.05 ± 0.288^c	19.8 ± 0.737^c

(Values are mean ± SEM of five animals). Significant difference at $p < 0.05$ (a: I vs IV, b: IV vs V, c: IV vs VI)
* 20 days withdrawal of the treatment
** 45 days withdrawal of the treatment

Antioxidant markers

Oral administration of EGME for 6 and 10 days did not affect the level of MDA and activities of SOD, Catalase and GPx compared with the controls. However significant increase in the level of MDA with significant decrease in the activities of testicular SOD, catalase and GPx compared with the controls were noticed in the mice treated with EGME for 10 days followed by 20 days withdrawal of treatment. The altered levels of MDA, SOD, catalase and GPx were restored in the mice received EGME for 10 days followed by 45 days withdrawal of the treatment as well as in the mice supplemented with WS (100 mg/kgBW/day) for 20 days following 10 consecutive days treatment with EGME (Table 3).

Table 3: Effect of ethanolic extract of *Ws* on testicular oxidative stress of EGME intoxicated mice.

Groups	MDA (nMoles/mg protein)	SOD (Unit/mg protein)	Catalase (µM/min/mg protein)	GPx (Unit/mg protein)
I: Control	0.203 ± 0.011	0.272 ± 0.021	17.243 ± 3.21	0.127 ± 0.015
II: EGME (600 mg/kgBW/day for 6 days)	0.347 ± 0.009	0.283 ± 0.024	16.259 ± 1.714	0.128 ± 0.020
III: EGME (600 mg/kgBW/day for 10 days)	0.333 ± 0.018	0.215 ± 0.031	17.244 ± 1.420	0.093 ± 0.020
IV: EGME* (600 mg/kgBW/day for 10 days)	3.910 ± 0.099^a	0.079 ± 0.015^a	5.935 ± 1.096^a	0.084 ± 0.002^a
V: EGME** (600 mg/kgBW/day for 10 days)	0.372 ± 0.023^b	0.273 ± 0.064^b	20.283 ± 2.607^b	0.139 ± 0.007^b
VI: EGME (600 mg/kgBW/day for 10 days) + *Ws* (100 mg/kgBW/day from 11th to 30th day)	0.428 ± 0.028^c	0.238 ± 0.027^c	13.899 ± 2.591^c	0.114 ± 0.002^c

(Values are mean ± SEM of five animals). Significant difference at $p < 0.05$ (a: I vs IV, b: IV vs V, c: IV vs VI).
* 20 days withdrawal of the treatment
** 45 days withdrawal of the treatment

DISCUSSION

Testis has an abundance of highly unsaturated fatty acid, hence is highly vulnerable to oxidative stress. The results of the present study indicate oxidative stress-induced impairment in sperm characteristics following EGME exposure and its prevention with the root extract of *Ws*.

Oxidative stress results in tissues due to an imbalance between the production and removal of reactive oxygen species

(ROS). The most prominent ROS are the superoxide anion (O2.-), hydrogen peroxide (H_2O_2), and the hydroxyl ion (OH.). They are playing a dual role as both deleterious and beneficial species and can be either harmful or beneficial to the living systems (Valko *et al.* 2007). ROS in low and limited concentration, play an important role in physiology of spermatozoa and more precisely in the acquisition of fertilizing ability by promoting the binding of spermatozoa to the zona pellucida and acrosome reaction (De Lamirande *et al.*, 1997) and also in normal testicular physiology (Boekelheide, 2005). The harmful effect of ROS occurs when there is an overproduction of ROS along with the deficiency of enzymatic and non-enzymatic antioxidant system. The major antioxidant enzymes in mammals are SOD, catalase, and GPX, which are expressed in the testis (Maiorino *et al.*, 2003; Ischi *et al.*, 2005). Earlier study has marked the involvement of ROS in the infertility due to defective spermatozoa function (Aitken *et al.*, 1987; Agarwal *et al.* 2014).

In the present study, no significant alterations were noticed in the oxidative status of the testis as well as the sperm characteristics in the epididymis 6 and 10 days after treatment with EGME. However, significant alterations were noticed in the same following administration of EGME for 10 days followed by their sacrifice 20 days after the treatment withdrawal. Further, significant reductions were also noticed in the percentage of epididymal spermatozoa motility and viability and count in the same, with significant increase in spermatozoa abnormalities. The results are consistent with the findings of earlier authors (Wang *et al.*, 2006; Isaac and Ebenezer, 2010). Isaac and Ebenezer (2010) has reported a significant decrease in SOD, Catalase and GPx activities with significant increase in the MDA level in the testis of the mice exposed to EGME (200 and 400 mg/kgBW/day) in drinking water for 14 weeks. The discrepancy between the present result and of Isaac and Ebenezer (2010) may be due to prolonged exposure of EGME in later studies while in the present study the mice were exposed with EGME only for 10 days. Further, the delayed impaired response as noticed in the present study clearly indicates that EGME not itself but its active metabolite, ethoxyacetic acid, formed as a result of metabolism of EGME, might have exerted oxidative stress 20 days after withdrawal of the treatment. Wang *et al.*, (2006) reported the inhibitory effect of EGME metabolite, ethoxyacetic acid on sperm motility. The delayed expression of acute effects of its other active metabolite, methoxyacetic acid is also well reported (Miller *et al.*, 1982). Induction of stress following EMGE treatment withdrawal again suggest the delayed response of EGME due to its conversion into methoxyacetic acid that might have damaged the spermatozoa which are highly susceptible to ROS because of their high content of polyunsaturated fatty acid, thus impairing the spermatozoal function.

Therefore, significant alterations noticed in the testicular oxidative stress could be correlated with the altered sperm parameters in 20 days treatment withdrawal mice, which clearly indicate the delayed impaired response of the testis to EGME even when administered for 10 consecutive days only. It appears that this toxicant might have retained in the testis, exerting its deleterious effects on the germ cells for several days after administration, the outcome of which is evidenced by significant alterations in the sperm indices. The inhibition of sperm motility, as noticed in the 20 days treatment withdrawal group, is accompanied by significant decline in the percentage of viable spermatozoa and increase in the percentage of abnormal spermatozoa.

Spermatogenic recovery is generally determined 3-4 weeks after treatment-induced inhibitions. Forty two days withdrawal of EGME treatment in the mice of group III showed significant recovery in the sperm parameters and oxidative stress markers. These recoveries reflect the reversible effects following exposure to EGME.

Since, the EGME adversely affected the epididymal sperm parameters and induced testicular oxidative stress following 20 days withdrawal preceded by 10 days treatment, therefore, it was planned to study the efficacy of root extract of *Ws* (100 mg/kgBW/day) in 10 days EGME-treated mice following its administration from 11th to 30th day. In this supplementation study it was found that, treatment with *Ws* significantly restored the activities of antioxidant enzymes, sperm motility, viability and counts along with significant decrease in the level of testicular MDA and sperm morphological abnormalities. Protective efficacy of *Ws* has also been reported in galactose-stressed mice (Patil *et al.*, 2012) and arsenic-induced testicular toxicity in the rat (Kumar *et al.*, 2015). Reports suggest the antioxidant property of *Ws* is attributed to the presence of Withaferin A (Glycowithanolides) and Sitoindosides VII-X (Bhattacharya *et al.*, 1997; Dhuley, 1998). In addition, the antioxidant efficacy of *Ws* is also attributed to the presence of other compounds such as polyphenols, flavonoids and vitamin-C (Visavadiya and Narasimhacharya, 2007; Udayakumar *et al.*, 2010). These compounds have been shown to reduce the levels of lipid peroxidation and oxidation of LDL (Tiwari, 1999; Jovanovic and Simic, 2000) by scavenging the hydroxyl and superoxide anions free radicals. Further, studies have demonstrated the beneficial effects of polyphenols and flavonoids which stimulate SOD and catalase gene transcription, thereby, increasing the antioxidant activities and reducing the lipid peroxidation level (Toyokuni *et al.*, 2003; Ranaivo *et al.*, 2004). Moreover, root enriched with ascorbic acid could readily scavenge ROS and can increase the testicular antioxidant activity, thereby, preventing the lipid peroxidation (Ray and Husain, 2002; Sowell *et al.*, 2004).

On the whole, it is suggested that the potential source of active compounds present in the root extract of *Ws* could have directly scavenged the free radicals, reduced the degradation of antioxidants and increased the biosynthesis of antioxidants through scavenging the excess amount of free radicals, thereby, increasing the bioavailability of antioxidants under oxidative stress conditions. This, further, might have improved the whole anti-oxidants system in the testis and thus the sperm characteristics in the epididymis.

CONCLUSION

It can be concluded that EGME exposure induces reversible delayed adverse actions through alterations in testicular oxidative stress and sperm indices. Further, the root extract of *Ws* is proved to be useful in preventing testicular oxidative stress and epididymal sperm characteristics. The investigations of molecular responses with *Ws* is required which may assist to elucidate the true impact and overall extent of recovery.

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