Original Research Article

SUPPRESSION OF FERTILITY IN MALE RATS BY HYDROETHANOLIC EXTRACT OF *ACHYRANTHES ASPERA*

Abu Hasnath Md Golam Sarwar[1,2], Beena Khillare[2], Sonu Chand Thakur[1] *

1. Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi-110025, India.

2. Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi-110067, India

ABSTRACT

The present study was designed to explore antifertility efficacy of *Achyranthes aspera* in male rats and to study different reproductive parameters related to male reproduction after *in vivo* exposure to understand the possible mechanism involved in it. Male rats were exposed to *Achyranthes aspera* hydroethanolic extract at doses of 100 and 200 mg/kg for 60 days. Histological studies revealed that *Achyranthes aspera* hydroethanolic extract caused degeneration of Leydig cells, spermatogenic disruption and shrinkage of seminiferous tubules in the testis. Seminal vesicle and prostate secretions were blocked and comparatively less population of spermatozoa were seen in the lumen of epididymis.   The serum testosterone level reduced significantly. The implantation sites in female rats were drastically reduced after treated males were mated with normal cyclic females. The level of non-enzymic and enzymic antioxidants like GSH, catalase, SOD, GPx, GR and GST were significantly lowered. Toxicity studies showed very minimal or no significant changes in the level of serum AST, ALT and creatinine of liver and kidney. It can be concluded that infertility in male rats could be due to impairment of reproductive performance as indicated by reproductive tract lesions, reduction in the level of testosterone and decrement in the levels of antioxidant enzymes.

Keywords: *Achyranthes aspera,* testosterone, histology, antioxidant enzymes, infertility

***Corresponding author*: Sonu Chand Thakur** Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi-110025, India.Tel: +91 11 26981717/4492 (0), Fax: +91 11 26983409; E-mail address: sonuchandthakur@gmail.com (S.C. Thakur)

Running Title: *Achyranthes aspera* suppresses fertility in male rats

The type of manuscript: Physical findings

INTRODUCTION

Achyranthes aspera Linn. (family Amaranthaceae), commonly known as Latjeera (Hindi) & Prickly Chaff tree (English) is widespread in the world as a weed, in Tropical Asia, Baluchistan, Sri Lanka, Africa, Australia and America. Traditionally, the plant is used in asthma and cough. It is pungent, antiphlegmatic, antiperiodic, diuretic, useful in oedema, dropsy and piles, boils and eruptions of skin etc. Principal constituents of *Achyranthes aspera* are alkaloids, betaine and achyranthine, identified from the whole plant [1].

Earlier studies conducted showed antifertility property of *Achyranthes aspera* [2]. The composite extract of *Achyranthes aspera* and *Stephania hernandifolia* reported to possess spermicidal activity in male rats [3]. It has been reported that a protein present in the ethnolic extract of *Achyranthes aspera* is responsible for the spermatoxicity and spermicidal activity in male rats [4,5].

Although these studies reported antifertility activity of *Achyranthes aspera* but the detail analysis of this plant regarding antifertility activity has not been done. Hence the present investigation has been carried out to investigate antifertility efficacy and to study different reproductive parameters related to male reproduction after *in vivo* exposure, to understand the possible mechanism involved in it.

MATERIAL AND METHODS

Chemical reagents

Citric acid, 5,5′-dithiobis (2-nitro-benzoic acid) (DTNB), 1-chloro-2,4-dinitro-benzene (CDNB), L-glutathione reduced, L-glutathione oxidized, nicotinamide adenine dinucleotide phosphate, and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents used were of analytical grade and purchased from Merck Ltd., India.

Animals

Experiments were performed on female Holtzman rats of 6-7 weeks of age (weighing 150-180g). These animals were procured from the Central Animal house Facility, National Institute of Health and Family Welfare (NIHFW), Munirka, New Delhi, India and housed separately in cages under standard conditions of 12h/12h day/night cycle, fed with standard rodent chow and water *ad libitum*. The environmental temperature and humidity maintained at 25°C ± 2 and 42% ± 5 respectively. The approval for experiment on animals was taken from Institutional Animals Ethics Committee of NIHFW (IAEC No. 168/1999)

Plant material collection and experimental design

The roots of *Achyranthes aspera* were collected from herbal garden of Jamia Hamdard, were authenticated by Dr MP Sharma, Department of Botany, Jamia Hamdard, Delhi, India. The roots of *Achyranthes aspera* was extracted thrice with 50% ethanol for 36 hours hours in an orbital shaker. The extract was filtered and concentrated *in vacuo* to get a green oily mass which is collected and stored at -20°C till further use. To investigate the effects of AAE, male rats were divided into three groups of 6 rats each and administered orally for 60 days.

Control: vehicle only (2% gum acacia).

Group I: 100 mg/kg body weight AAE suspended in vehicle.

Group II: 200 mg/kg body weight AAE suspended in vehicle.

After last oral dose, the animals were sacrificed by cervical dislocation after mild anesthesia. For hormone analysis and biochemical tests blood was collected from dorsal aorta using a heparinized syringe attached with 21-gauge syringe needle. Male reproductive organs (testis, epididymis, vas deferens, seminal vesicle, prostate gland) were processed for histology, enzymic activity.

Hormone analysis

The circulating serum levels of testosterone were determined by ELISA kit (Uscn Life Science Inc.) as per the instructions laid by the manufacturer.

Histological evaluation   For
histological observations, testis, epididymis, seminal vesicles and prostate gland were fixed in buffered
formaldehyde solution, embedded in paraffin and serially sectioned at 5 µm thickness. Sections were
stained with hematoxylin and eosin for microscopic observation.

Anti-implantation activity

For implantation study, AAE treated male rats were caged with fertility proven females at a ratio of 1:2 and
vaginal smear was assessed each day for the presence of copulatory plug. The presence of copulatory
plug was taken as evidence of fertile mating and was counted as day 1 of pregnancy. The mated females
were lapratomized on 11th day of pregnancy and uterus were examined to determine the number of
implantation sites control and treated groups. Mating that did not lead to successful pregnancy was
considered as infertile mating.

Estimation of antioxidant activity

To prepare tissue homogenate, ovaries were dissected out, rinsed quickly in cold saline solution and
homogenized in 0.05 M Tris-HCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. An aliquot of the
homogenate (0.5 ml) was used for assaying soluble sulfhydryl group (-SH) while the remainder was
centrifuged at 1000 × g for 10 min. The resultant supernatant was transferred into pre-cooled centrifugation
tubes and centrifuged at 12,000 × g for 30 min. The supernatant (cytosolic fraction) was used for assaying
the antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase
(GPx), glutathione reductase (GR) and glutathione S-transferase (GST). The fraction was stored at -80ºC.

a) **Estimation of acid soluble sulfhydryl (–SH) group**

The level of acid soluble sulfhydryl group was estimated as total non-protein sulfhydryl group as
reported by Singh et al. (2000) [6]. Reduced glutathione was used as a standard to calculate µM of –
SH content/g tissue.

b) **Determination of SOD, CAT, GPx, GR and GST activity**
The cytosolic SOD, CAT, GPx, GR and GST activities were determined spectrophotometrically at 37°C
[6]. Enzyme activity was expressed as units/mg protein.

**Assay of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and creatinine
activity**

The method of Reitman and Frankel was used to determine AST and ALT activities. Plasma creatinine
concentrations were measured using the method of the Jaffe color reaction [7].

Statistical analysis   Results
are expressed as mean ± standard deviation of n = 6 animals per experimental group. Statistical analysis
was performed using GraphPad Prism software version 6.00. Differences between the means of
experimental and control groups were analyzed by one-way ANOVA followed by Tukey-Kramer post-hoc
test. The level of significance was accepted at $p < 0.05$.

RESULTS

Serum testosterone level
Significant difference in the circulatory level of testosterone was observed in the   AAE treated animals as
compared to vehicle treated control. Testosterone level declined gradually and was significantly lower at
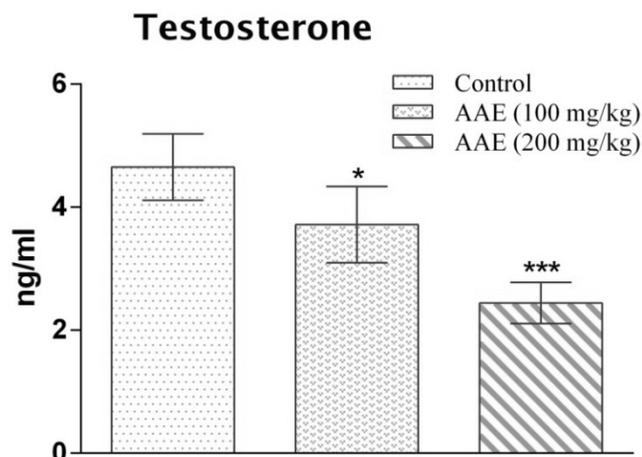100 mg/kg ($p < 0.05$) and 200 mg/kg ($p < 0.001$) dose (Fig. 1).

Fig.1. Effect of AAE treatment on the serum levels of testosterone

Data are mean ± S.E.M. of 6 replicates. Significant difference at * $p < 0.05$ level, *** $p < 0.001$ level, when compared with control group.

Histological study of reproductive organs

Testis

Control animals showed normal cellular arrangement in the seminiferous tubules (Fig. 2A). Histology of the testis was not altered in the animal treated with 100 mg/kg dose (Fig. 2B). On the other hand, seminiferous tubular shrinkage and Leydig cells degeneration were observed in 200 mg/kg dose treated rat testis (Fig. 2C). Seminiferous tubules revealed spermatogenic disruption and loosened epithelium. There was also reduction in the number of germ cells in the seminiferous tubules.

Epididymis

In control rats the lumen of ductules were fully packed with spermatozoa (Fig. 2D). The population of spermatozoa was not much affected at 100 mg/kg dose (Fig. 2E). However, most of the lumen of ductules at higher dose had either comparatively less population of spermatozoa or were devoid of spermatozoa in contrast to controls (Fig. 2F).

Seminal vesicle

Secretion in the lumen of the seminal vesicle was very less and the luminal space was reduced in the rats treated with 200 mg/kg dose (Fig. 2I) as compared to controls (Fig. 2G). At 100 mg/kg dose the secretion in the lumen was not much affected (Fig. 2H).

Prostate gland
The alveolar lumen of the prostate was devoid of secretion at the higher dose (200 mg/kg) studied (Fig. 2L). The mucosal folds of the lumen were invaded into the scanty lumen. The prostate at lower dose (Fig. 2K) caused inhibition of secretion in some of the alveolar lumens when compared to its control (Fig. 2J).
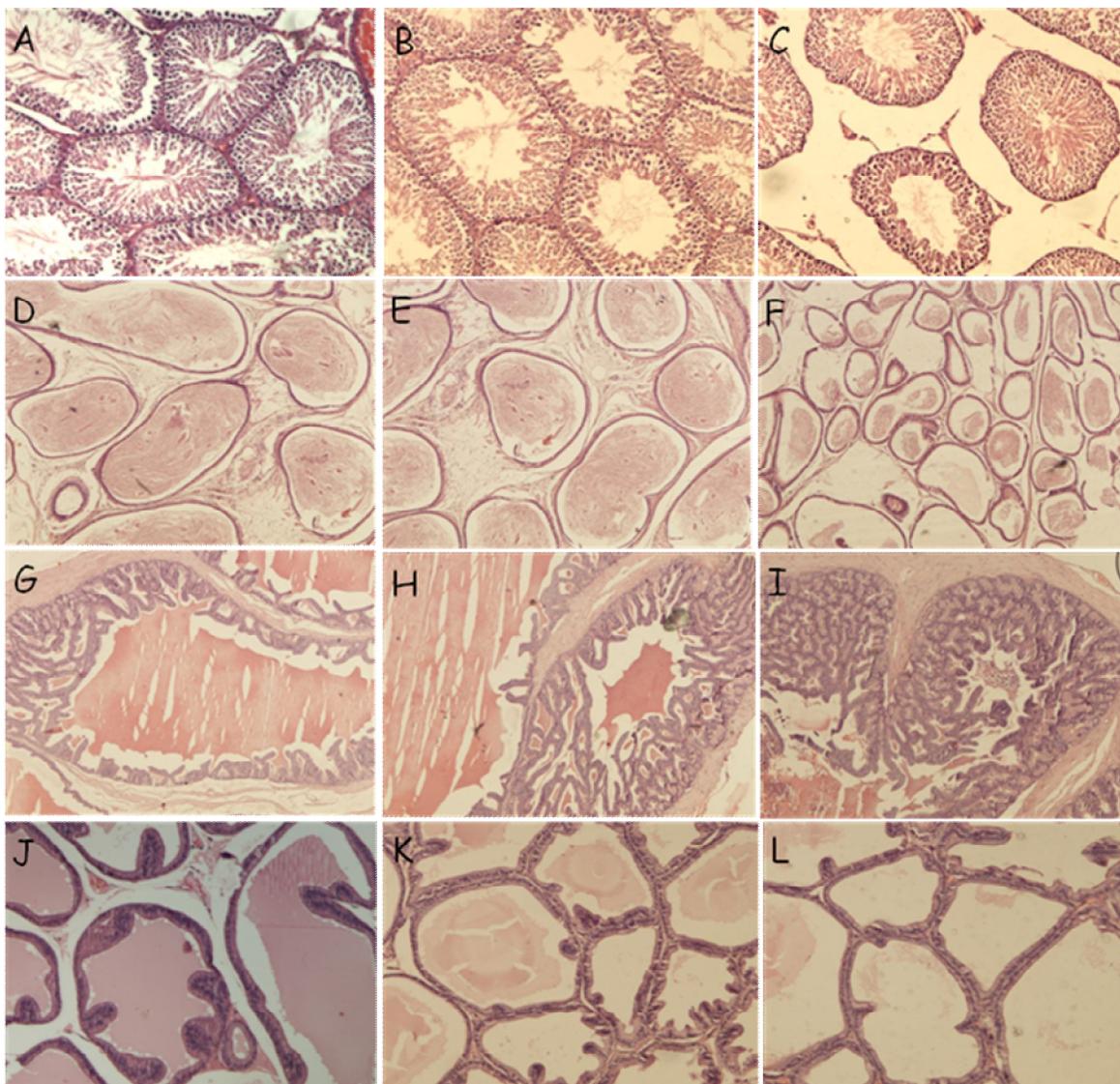
Fig. 2. Representative photomicrographs of rat testis, epididymis, seminal vesicle and prostate sections: A. Section of testis from control rat showing normal spermatogenesis **B.** Section of testis treated with 100 mg/kg dose showing normal cellular arrangement **C.** Section of testis treated with 200 mg/kg dose showing shrinkage of seminiferous tubules and spermatogenic disruption **D.** Section of epididymis from control rat showing lumen of ductules fully packed with spermatozoa **E.** Section of epididymis treated with 100 mg/kg dose showing spermatozoa packed in the lumen of ductules **F.** Section of epididymis treated with 200 mg/kg dose showing comparatively less population of spermatozoa **G.** Section of seminal vesicle from control and **H.** treated rat with 100 mg/kg dose showing normal secretion **I.** Section of seminal vesicle treated with 200 mg/kg dose showing less secretion **J.** Section of prostate from control rat showing alveolar lumen fully packed with secretion **K.** Section of prostate from 100 mg/kg dose treated rat showing less secretion in the lumen **L.** Section of prostate from 200 mg/kg dose treated rat showing inhibition of secretion in the lumen (400X magnification).

Effect on fertility and implantation

After AAE treatment of 30 days, male rats were mated with fertile female rats. AAE affected fertility rate of male rats. AAE promoted infertile mating in treated animals in a dose dependent manner. The number of implantation site was also reduced significantly at both the doses of 100 ($p < 0.5$) and 200 mg/kg ($p < 0.001$) (Fig.3).
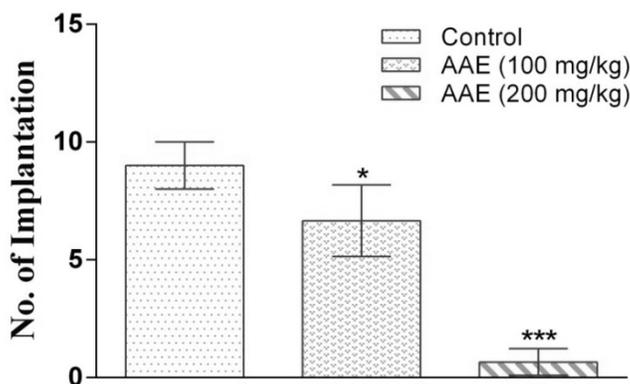

Fig.3. Effect on the implantation of female rats after mating with AAE treated male rats

Each value represents the mean ± S.D. of n = 6 animals per group.
$^*p < 0.05$, $^{***}p < 0.001$ versus control + vehicle group.

Enzymic and nonenzymic antioxidant levels

A) GSH Assay

We assessed the effects of AAE treatments on the level of reduced glutathione measured as acid soluble sulphydryl group (–SH) in all the experimental rats. Fig.4 summarizes the testis, seminal vesicle and prostate content of GSH. AAE at the dosage of 100 mg/kg caused a significant decrease in the levels of reduced glutathione by 1.13 ($p < 0.05$) and 1.15 fold ($p < 0.05$) in the testis and prostate, respectively. There was no significant decrease in the glutathione level in the seminal vesicle. The rats treated with AAE (200 mg/kg) showed a further significant decrease in the non-enzymic antioxidant level to 1.45 ($p < 0.001$), 1.2 ($p < 0.05$) and 1.6 ($p < 0.001$) fold in testis, seminal vesicle and prostate, respectively.

B) Level of antioxidant enzymes

The antioxidant enzymes total superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) activities were determined in the testis, seminal vesicle and prostate gland of all the experimental rats. AAE treated rats were compared with age matched healthy control rats. The results are presented in Fig.4.

At 200 mg/kg dosage of AAE, the levels of SOD, CAT, GPx, GR and GST were decreased by 1.84 ($p < 0.05$), 1.74 ($p < 0.01$), 1.40 ($p < 0.05$), 1.42 ($p < 0.05$), and 1.50 fold ($p < 0.05$) in the prostate gland, respectively. Barring catalase, which showed significant ($p < 0.05$) decrease, there was no significant difference found with respect to SOD, GPx, GR and GST activities at 100 mg/kg of AAE.

The seminal vesicle from the rats treated with AAE (200 mg/kg) showed a decline in the level of SOD, CAT, and GST by 1.68 ($p < 0.01$), 1.6 ($p < 0.01$) and 1.36 fold ($p < 0.05$). The level of GPx and GR also showed

similar decrease of 1.33 ($p < 0.01$) fold. There was no significant reduction in the level of antioxidant enzymes, observed at 100 mg/kg dose groups.

The activity of GPx and GR was decreased by almost 1.25-fold ($p < 0.05$) in the testis from rats treated with 200 mg/kg of AAE while a 1.45-fold decrease ($p < 0.01$) was observed in the activity of SOD, CAT and GST. No effect of treatment was observed in the testis of rats treated with 100 mg/kg of AAE.
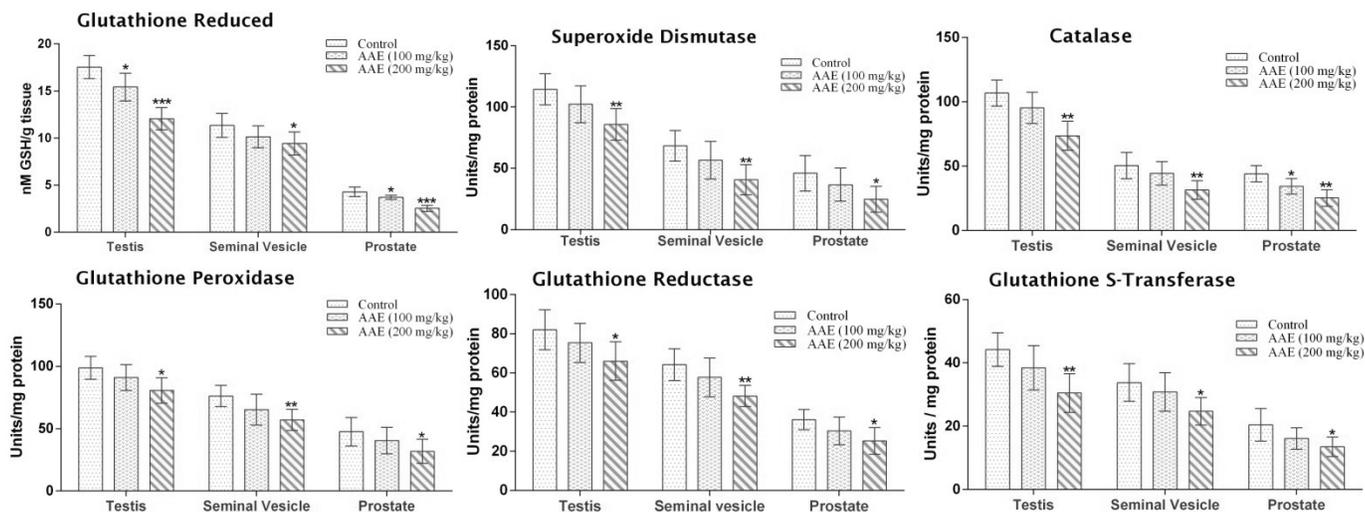

Fig.4. Effects of AAE (*Achyranthes aspera*) on the levels of different non-enzymic and enzymic antioxidants measured in the ovary of rats from each group. Results of

GSH expressed as nM GSH/g tissue, antioxidant enzymes (SOD, CAT, GPx, GR, GST) are expressed in units/mg protein.
Each value represents the mean ± S.D. of n = 6 animals per group.
$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control + vehicle group.

Serum marker enzymes

There was no significant change in the AST enzyme at both the doses of AAE. The ALT enzyme level was increased significantly only at higher dose of 200 mg/kg ($p < 0.05$). However at lower dose the increment was not significant (Fig. 5).

Similarly at lower dose of AAE, the level of serum creatinine concentrations was not elevated significantly but at higher dose of 200 mg/kg the serum concentrations of creatinine was increased significantly as compared to vehicle control (Fig. 5).
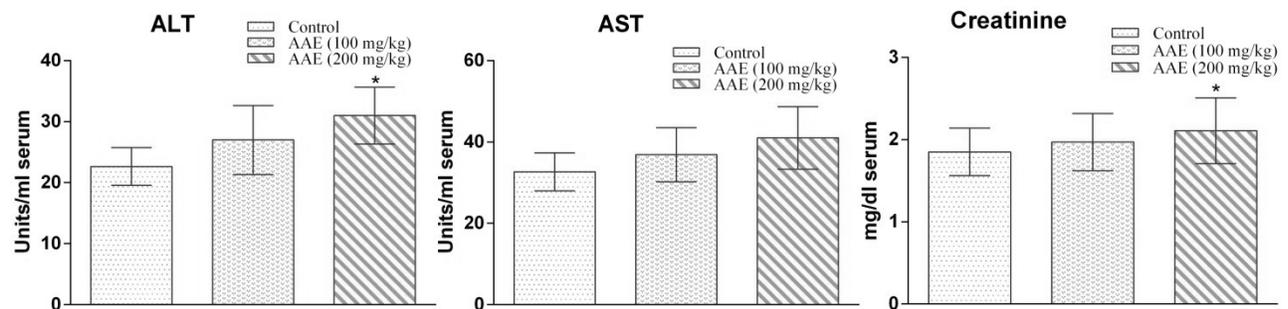
Fig.5. Serum levels of Alanine transaminase (ALT), Aspartate transaminase (AST) and Creatinine after AAE administration.
Each value represents the mean ± S.D. of n = 6 animals per group.
*$p < 0.05$ versus control + vehicle group.

DISCUSSION

Testosterone helps in maintenance of spermatogenesis and is responsible for the growth, structural integrity and functional activities of accessory sex organs as well as it help in the maintenance of spermatogenesis. During spermatogenesis it plays important role in the development and maturation of sperm, so decrease in the level of testosterone may decrease the quality of sperm [8]. Many studies have shown that testosterone withdrawal from the rat testis results in increased germ cell apoptosis [9].

In the present study AAE administration reduced testosterone level and degenerated Leydig cells in testis. Since Leydig cells produces testosterone in testis, it is possible that AAE treatment might have modulated Leydig cell function which disrupted steroid production in treated animals. Further reduced testosterone level might have disrupted spermatogenesis and induced infertility in treated rats.

The production of mature sperm with normal structure and function is the benchmark of male fertility. This is achieved by normal spermatogenesis and spermiogenesis in the testis, functional maturation during epididymal transit as well as seminal plasma contributed by normally secreting accessory sex glands. In the present study, the histology of the accessory sex glands showed very less secretion in the lumen and there was less population of spermatozoa in the tubules of epididymis which most likely account for the reduction in fertility in male rats. These accessory sex glands provide sperm with a nutritious medium for gamete transfer and secrete antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase as well as free radical scavengers such as Vitamins C and E, hypotaurine etc [10].

A well-balanced amount of ROS plays an essential role in maintaining sperm structure, genomic and functional integrity, as well as preserving the antioxidants in secretions of the male reproductive tract [10]. The increased susceptibility of spermatozoa to oxidative stress may be associated with a variety of factors. These include, among others, the lack of cytoplasm in mature spermatozoa, which could be associated with a lower overall antioxidant capacity, compared to somatic cells. The ROS cause damage to sperm, mitochondrial and other cytoplasmic organelle membrane structures through peroxidation of phospholipids, proteins and nucleotides [11].

Glutathione plays an important role in normal function as an antioxidant. The important functions include: the maintenance of the sulfhydryl groups in proteins; translocation of amino acids across cell membranes; the detoxification of foreign compounds; and the biotransformation of drugs.

ROS produce oxidative stress by decreasing enzymatic defenses [12]. Superoxide dismutase constitutes the first line of coordinated enzymatic defense against ROS by dismutating $O_2 \bullet -$ into O_2 and H_2O_2. It has been reported that the addition of SOD to sperm in culture protects them from oxidative attack [13]. Some investigators have also reported minor reductions in seminal plasma SOD activity in infertile men [14,15]. Catalase and glutathione peroxidase are most crucial for detoxifying H_2O_2, thereby preventing the generation of hydroxyl radical by the Fenton reaction. CAT and GPx also protect SOD against inactivation by H_2O_2. Reciprocally, SOD protects CAT and GPx against inhibition by superoxide anion. Some of the studies have shown a relation between deficient seminal catalase activity and male infertility [14,15,16].

In the present study, AAE administration decreased the activities of SOD, CAT, GPx, GR, GST and GSH levels in   testis, seminal vesicle and prostate. The reduction in the activity of CAT observed in the present study may reflect inability of cells to eliminate H_2O_2 produced by AAE which may be due to enzyme inactivation caused by excess production of ROS.

GPx is primarily responsible for H_2O_2 removal in testicular mitochondria that does not contain catalase. GPx plays a crucial role in scavenging peroxyl radicals and thereby maintains functional integrity of the cell membrane, spermatogenesis, sperm morphology, and motility [17]. The continued activity of GPx depends on the regeneration of reduced glutathione by glutathione reductase (GR). Selective inhibition of GR reduces the availability of reduced glutathione for maintaining GPx activity, thereby exposing sperm to oxidative stress [18]. The coordinated activity of GPx, GR and glutathione clearly play a pivotal role in protecting sperm from oxidative attack. Glutathione-S-transferase metabolizes xenobiotics by conjugating with GSH. In fact, GST catalysed conjugation of GSH with exogenous compounds and endogenous metabolites such as 4-hydroxynonenal is regarded as major cellular defense mechanism against toxicity [19].

Thus, the balance of these enzyme systems may be essential to get rid of superoxide anion and peroxides generated in subcellular compartments of the testis, prostate and seminal vesicle. The reduced activity of GPx in the organ homogenates observed in this study may partly be due to lack of the substrate GSH.

The most common markers for hepatic toxicity are serum AST and ALT. When the liver is damaged by any cause, the levels of these proteins are increased rapidly [20]. Serum creatinine is used clinically to detect and evaluate kidney injury and chronic kidney disease [21,22]. Therefore the level of creatinine is significant to any kind of renal toxicity. This study demonstrates that oral administration of AAE increases the level of serum ALT, AST and creatinine significantly at higher dose, however there is no significant damage seen in the histoarchitechture of both liver and kidney.

Declaration of Interest

The authors report no declarations of interest.

Acknowledgement

REFERENCES

1. Varuna KM, Khan MU, Khan PK. Review on *Achyranthes Aspera. J Pharm Res.* 2010;3(4):714-7.
2. Sandhyakumary K, Boby RG, Indira M. Impact of feeding ethanolic extracts of Achyranthes aspera Linn. on reproductive functions in male rats. *Indian J Exp Biol.* 2002;40(11):1307-9.

3. Paul D, Bera S, Jana D, Maiti R, Ghosh D. *In vitro* determination of the contraceptive spermicidal activity of a composite extract of *Achyranthes aspera* and *Stephania hernandifolia* on human semen. *Contraception.* 2006;73(3):284-8.

4. Anuja MNMK, Nithya RNSA, Rajamanickam C, Madambath I. Spermatotoxicity of a protein isolated from the root of *Achyranthes aspera*: a comparative study with gossypol. *Contraception.* 2010;82(4):385-90.

5. Anuja MM, Nithya RS, Swathy SS, Rajamanickam C, Indira M. Spermicidal action of a protein isolated from ethanolic root extracts of *Achyranthes aspera*: An *in vitro* study. *Phytomedicine.* 2011;18(8):776-82.

6. Singh RP, Padmavathi B, Rao AR. Modulatory influence of *Adhatoda vesica* (*Justicia adhatoda*) leaf extract on the enzymes of xenobiotic metabolism, antioxidant status and lipid peroxidation in mice. *Mol Cell Biochem.* 2000;213(1-2):99-109.

7. Tymecki L, Korszun J, Strzelak K, Koncki R. Multicommutated flow analysis system for determination of creatinine in physiological fluids by Jaffe method. *Anal Chim Acta.* 2013;787:118-25.

8. Kefer JC, Agarwal A, Sabanegh E. Role of antioxidants in the treatment of male infertility. *Int J Urol.* 2009;16(5):449-57.

9. Nandi S, Banerjee PP, Zirkin BR. Germ cell apoptosis in the testes of Sprague Dawley rats following testosterone withdrawal by ethane 1,2-dimethanesulfonate administration: relationship to Fas? *Biol Reprod.* 1999;61(1):70-5.

10. O WS, Chen H, Chow PH. Male genital tract antioxidant enzymes--their ability to preserve sperm DNA integrity. *Mol Cell Endocrinol.* 2006;250(1-2):80-3

11. Agarwal A, Nallella KP, Allamaneni SS, Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomed Online.* 2004;8(6):616-27.

12. Griveau JF, Le Lannou D. Influence of oxygen tension on reactive oxygen species production and human sperm function. *Int J Androl.* 1997;20(4):195-200.

13. Kobayashi T, Miyazaki T, Natori M, Nozawa S. Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Hum Reprod.* 1991;6(7):987-91.

14. Alkan I, Simsek F, Haklar G, Kervancioglu E, Ozveri H, Yalcin S, et al. Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. *J Urol.* 1997;157(1):140-3.

15. Sanocka D, Miesel R, Jedrzejczak P, Chelmonska-Soyta AC, Kurpisz M. Effect of reactive oxygen species and the activity of antioxidant systems on human semen; association with male infertility. *Int J Androl.* 1997;20(5):255-64.

16. Zini A, Garrels K, Phang D. Antioxidant activity in the semen of fertile and infertile men. *Urology.* 2000;55(6):922-6.

17. Calvin HI, Turner SI. High levels of glutathione attained during postnatal development of rat testis. *J Exp Zool.* 1982;219(3):389-93.

18. Williams AC, Ford WC. Functional significance of the pentose phosphate pathway and glutathione reductase in the antioxidant defenses of human sperm. *Biol Reprod.* 2004;71(4):1309-16.

19. Cheng JZ, Singhal SS, Sharma A, Saini M, Yang Y, Awasthi S, et al. Transfection of mGSTA4 in HL-60 cells protects against 4-hydroxynonenal-induced apoptosis by inhibiting JNK-mediated signaling. *Arch Biochem Biophys.* 2001;392(2):197-207.

20. Sheth SG, Flamm SL, Gordon FD, Chopra S. AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. *Am J Gastroenterol.* 1998;93(1):44-8.

21. Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, Warnock DG, et al. Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. *Crit Care.* 2007;11(2):R31.

22. K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease. *Am J Kidney Dis*. 2003;42(4 Suppl 3):S1-201.