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An experimental study to demonstrate the extents of reversible changes after withdrawal of allethrin based mosquito coil smoke exposure along with the possible protective role of vitamin C on induced testicular changes in male wistar Albino rats

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Abstract: ***- This study was an attempt to delineate the effect of allethrin based mosquito coil exposure on male fertility of wistar albino rats. This also includes two months of discontinuation of exposure to know whether there is any reversibility of changes. We also studied the role of an antioxidant (Vitamin C) in ameliorating the effect caused by the coil.The animals in our study were randomized into four groups: group I served as control rats; group II, III and IV received mosquito coil smoke 8 hours a day, 7 days a week for 12 week. Group IV rats co-administered oral supplementation of Vit. C (20 mg/kg body weight) once in a day for the same time period while group III animals were further kept for 8 weeks without any exposure to demonstrate withdrawal effect. Mean testicular weight was maximum in Group IV(1.83±0.30 gm) followed by group I(1.56±0.19gms), group III(1.22±0.42 gms) and group II(0.64±0.09 gm). Mean sperm count ranged from 83.75±9.61 units in Group II to 100.0±16.68 units in Group III, 130.7±14.14 units in Group IV and 157.7±7.92 units (Group I). Mean % abnormal sperm morphology was maximum in Group II (36.95±7.87%) followed by Group III (30.66±9.59%), Group IV (11.97±2.74%) and Group I (5.37±1.28%). Statistically, this difference was significant (p<0.001). This study demonstrates different types of abnormal sperm morphology. This study has its own merit that it also depicts the possible improvement after discontinuation of exposure and vitamin C supplementation.***

Keywords *- Allethrin exposure, withdrawal effect, Sperm morphology, Male fertility, Vitamin C supplementation.*

Introduction

Male infertility is one of the major health problems in our society and has the important demographic implications worldwide. According to WHO estimate nearly 60 to 80million couples are affected by infertility annually and out of these 40% cases accounts for male infertility (Rutstein and Iqbal. 2004). In the last few years there has been considerable decrease in quality and quantity of sperm count. Many potentially hazardous chemicals have been released into the environment at an alarming rate, and their exposure to humans has become inevitable. Most environmental chemicals are hormonally active compounds and target the endocrine system causing reproductive anomalies. Environmental hazards to male reproductive system were revealed years ago with the discovery of pyrethroid. These pyrethroids are group of insecticides designed to impact immune, reproductive, or nervous system of insects. Pyrethroids are the synthetic derivatives of naturally occurring pyrethrin (Casida 1980), derived from dried and powdered flower of Chrysanthemum cinerarifolium and Tanacetum cinerariifolium. Most pyrethroids contain cyclopropane carboxylic acid moieties linked to aromatic alcohols through a central ester (or ether) bond (Wolansky and Harill 2007). On the basis of structure, pyrethroids are of type I and II. Type I pyrethroid contain ester bond with a central linkage without a cyano group. These include permethrin, allethrin, tetramethrin and fenothrin. Type II has a cyano group at the carbon of ester linkage. It includes cypermethrin, deltamethrin and fenvalerate. Use of pyrethroid is not only limited in agricultural field but it gained its importance in controlling household insects. Mosquitoes are important vector of many tropical diseases. Till date due to unavailability of any successful vaccine for these vector borne diseases, protection from mosquito bite is the only method for prevention and control of diseases. Control of these mosquito borne diseases rely solely on the use of various pyrethroid, and mosquito coil is the only option left in rural areas as it is cheaper and easily available (Mulla et al. 2001). One of the most commonly used pyrethroid in mosquito coil in India is allethrin. However this allethrin based coil not only targets insects but other mammalian species in vicinity. Therefore, the evaluation of toxic potential of allethrin is important for the risk assessment of human beings ordinarily exposed to these substances.

Material and Method

Experimental Rats:

A total of 42 healthy male albino rats of the age between 2-3 months weighing between 250-300 grams were obtained from the animal house of Indian Institute of Toxicology and Research, Lucknow. Ethical clearance was obtained from Animal Institutional Ethical Committee, King George's Medical University. After procurement of rats they were allowed to acclimatize for 2 weeks and were fed freely on standard pellet diet 5gm/rat/day. Relatively constant environmental conditions were maintained with proper aeration and good source of light (12hours light-12hours dark and 24 degree C ± 30 degree C). Food and water were provided ad libitum. Rats were kept in proper hygienic condition and care was delivered in accordance with the guidelines given by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Animals were divided into 4 groups

1) Group I – total of 12 rats with no exposure served as control group
2) Group II - total of 12 rats with 8 hours exposure to mosquito coil smoke 6 days in a week for 12 weeks.
3) Group III- total of 8 rats with 8 hours exposure to mosquito coil smoke 6 days in a week for 12 weeks and then kept for 8 weeks without exposure
4) Group IV-- total of 10 rats with 8 hours exposure to mosquito coil smoke 6 days in a week for 12 weeks along with oral supplementation of ascorbic acid (vitamin C) in the dose of 20 mg/kg body weight once in a day for the same time.

Mosquito coil exposure

Animals from experimental group II, III and IV were kept in a room of dimension 9.5 feet×9feet×9feet having proper cross ventilation. Rats were allowed to inhale allethrin based mosquito coil smoke by burning it for 8 hours (9:00 AM -5:00 PM). Each coil measured 15 cm in diameter and 12 gm in weight and contained 0.1%w/w of d-trans allethrin.

Treatment with ascorbic acid

Commonly available Vit.C tablets in the name of Limcee, manufactured by Abbott Healthcare Pvt Ltd (AHPL) were used. A fresh aqueous solution of vitamin C was prepared by dissolving one vitamin C tablet of 500 mg in 10 ml of water. Freshly prepared solution was orally administered with the help of feeding canula to rats of experimental group IV at dose level of 20mg/kg wt. (twice as the human recommended dose of 10 mg/kg body weight) (Kahn and Sinha 1993).

Animal Sacrifice and Sample Collection:

Rats from group I, II and IV were sacrificed after 12 weeks of exposure period, whereas rats from group III were sacrificed after further 8 weeks of cessation from mosquito coil smoke. Rats were anaesthetized by using chloroform. Thereafter they were spread on their back and all four limbs and were held by pinning them on the wax coated dissection tray. Then the incision was made on the thoracoabdominal part and the heart was exposed. The incision was extended down into the scrotal region and both the testis was taken out along with epididymis after tying the vasdeferens. Epididymis was separated from the testis and testis was washed with normal saline and weighed. Sperms were taken out by mincing the epididymis into 1 ml normal saline for sperm count and sperm morphology.

Preparation of slide for sperm morphology

Sperms were taken out by mincing the epididymis into 1 ml normal saline. One drop of this normal saline containing sperms was spread on a glass slide and was fixed in absolute alcohol. The sperm cells were evaluated with the aid of light microscope.

Method of sperm count:

The epididymal sperms were counted by using Neubauer chamber. Normal saline containing epididymal sperms was drawn to the 0.5 mark of a WBC pipette. Then the semen diluting fluid (soda bi-carbonate) was drawn to the 11 mark and mixed well.

The improved Neubauer chamber was loaded and sperms were allowed to settle for about 5 minutes. Sperms were then counted in the four corner squares.

The formula to count the number of sperms/ml of semen

Sperms/ml of semen= (sperms counted in 4 square ×10×20×1000)/4

Results

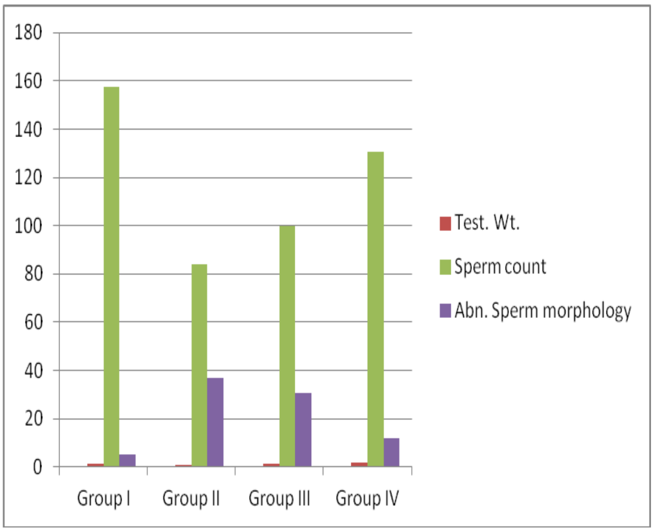
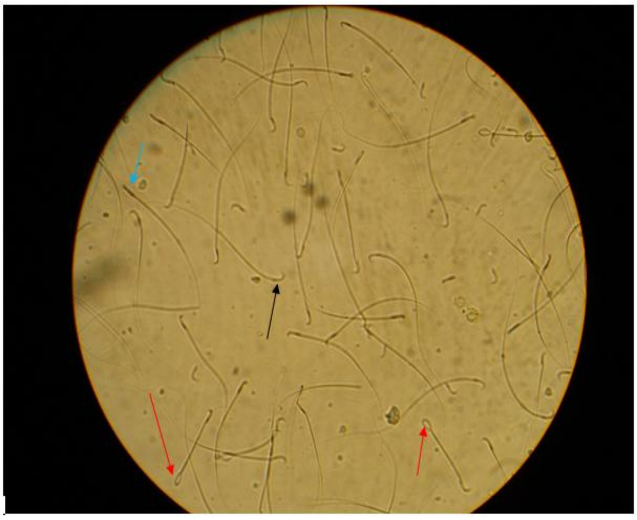


Figure 1 Comparison of testicular parameters in different groups

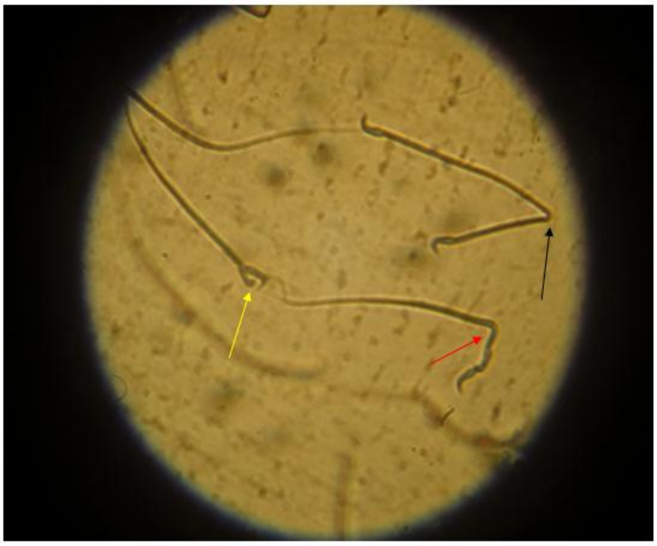
As shown in Table1 mean testicular weight ranged from 0.64±0.09 gm (Group II) to 1.83±0.30 gm (Group IV). Mean testicular weight in Groups I and III was 1.56±0.19 and 1.22±0.42 gms. Statistically, there was a significant difference among groups. Mean sperm count ranged from 83.75±9.61 units (Group II) to 157.7±7.92 units (Group I). Mean sperm count was 100.0±16.68 units in Group III and 130.7±14.14 units in Group IV. Statistically, this difference was significant ($p<0.001$).Mean % abnormal sperm morphology was maximum in Group II (36.95±7.87%) followed by Group III (30.66±9.59%), Group IV (11.97±2.74%) and Group I (5.37±1.28%) respectively, thereby showing a significant intergroup difference ($p<0.001$). Between Groups I and II, for all the three parameters, a significant difference was observed ($p<0.001$) (Table2.1). Mean testicular weight and sperm count was significantly higher in Group I as compared to Group II whereas mean % abnormal sperm morphology was significantly lower in Group I as compared to Group II (Table2.1 and figure 1).Between Groups I and III, a significant difference was observed for mean sperm count and % abnormal sperm morphology (Table2.1). Mean sperm count in Group I was higher whereas mean % abnormal sperm morphology was lower as compared to that in Group III (Table2.1 and figure 1).Between Groups I and IV, a significant difference was observed for % abnormal sperm morphology only. Mean % abnormal sperm morphology was significantly lower in Group I as compared to that in Group IV (Table2.1 and figure 1). Between Groups II and III, a statistically significant difference was observed only for testicular weight which was found to be significantly lower in Group II as compared to Group III (Table2.2 and figure 1). Between Groups II and IV and Groups III and IV, a significant difference was observed for all the three parameters. Mean testicular weight and mean sperm count was found to be significantly higher in Group IV as compared to both the other groups whereas mean abnormal sperm morphology was found to be significantly lower in Group IV as compared to both the other groups (Table2.2 and figure 1). Sperm with different type of abnormal morphology were encountered. The most common abnormality is head and tail deformity. Sperm with head abnormality included headless sperm (figure 4) and double head (figure 3) being the most common. Other forms of head abnormality were amorphous head (figure. 2) and banana head (figure 5). Tail abnormality included absent tail (figure 3), double tail (figure 5) bent tail (figure 4) and looped tail (figure 2 and 4).

Figure 2 Photomicrograph showing different types of sperm morphology



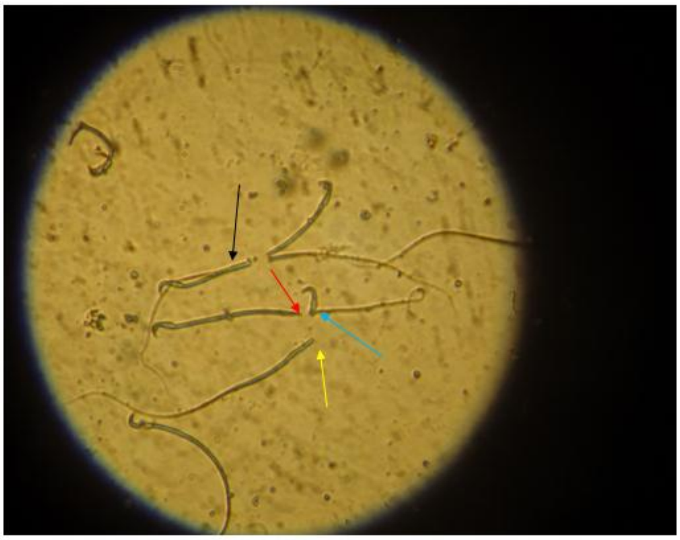
Black arrow indicating normal sperm
Red arrow indicating sperm with looped tail
Blue arrow indicating sperm with amorphous head (200X)

Figure 3 Photomicrograph showing different types of sperm morphology



Yellow arrow-sperm with double head
Red arrow-sperm with bent middle piece
Black arrow-sperm with fused middle piece and absent tail (400X)

Figure 4 Photomicrograph showing different types of sperm morphology



Black arrow- bent tail
Red arrow-absent tail
Yellow arrow-absent head
Blue arrow-bent middle piece with looped tail.
(400X)

Discussion

In countries where malaria and other mosquito borne diseases are endemic and where there is no effective program to combat them, use of parathyroid in the form of mosquito coil is a very regular practice. These pyrethroids interfere with normal reproductive parameters and result in male infertility. A number of studies have shown that these pyrethroids have devastating effect on testicular function and male germ line (Perry 2008). Since the weight of the testis is largely dependent on the mass of differentiated spermatogenic cells, testis weight has been used as a measure of spermatogenesis (Schlappack et al. 1988). It is logical; therefore, that a reduction in the weight of the testis has been shown to occur with loss of germ cells. It is evident from figure1 that there was significant decrease in weight of testis in exposure group II. Akunna et al (2013) also noted a significant decrease in weight of testis following exposure to mosquito coil and explained it due to the testiculotoxic nature of allethrin based mosquito coil. Similar results were obtained by Sakr and Azab (2001) by exposing rats to tetramethrin via inhalation and by Kalender et al (2006) and Kilian et al (2007) by exposing animals with different pyrethroid compounds like deltamethrin, fenvalerate and diazinon. These results are also in agreement with the report of (Garba et al. 2007) involving pyrethroid based mosquito coil exposure to animal models, showing a significant decrease in the testis weight and volume. Decrease in weight of testis may be due to apoptosis of spermatogenic cells following exposure to allethrin. Allethrin induces the production of lipid peroxide leading to oxidative degenerative changes in the cell and inhibition of mitochondrial action and eventually causing cell death. Various studies have shown that allethrin induces toxicity via oxidative stress by generation of free radical and reactive oxygen species (ROS) (Sanvidhan et al. 2006; Mathur 2008). Alhazza and Bashandy (1988) reported a loss in testicular weight and volume in pyrethroid exposed rats which were as a result of degenerative changes in the seminiferous epithelium. In group III there was increase in testis weight (figure 1) depicting regeneration of spermatogenic cells following cessation of exposure. Rats that were co-administered with Vit. C, there was significant increase in testis weight as shown in figure 1.This may be because of the antioxidant mechanism of ascorbic acid which prevents oxidative damage to spermatogenic cell thus minimizing their apoptosis. It was in consistent with the study of Turki et al (2013) in which treatment of cypermethrin along with vitamin E which is also an antioxidant, caused a significant increase in testis weight.

Sperm count

Sperm count is one of the most sensitive tests for assessment of spermatogenesis and fertility. Any factor that disturbs the homeostasis mechanism of male reproduction will definitely alter the sperm count. A decrease in sperm count in men over the last few decades is correlated with a steady increase in use of pesticides and other environmentally active chemicals (Carlsen et al. 1995). Pyrethroid exposure in various animals has been reported to decrease sperm count (Kamijima et al. 2004). In the present study, a significant reduction in total sperm count was observed after exposure to mosquito coil. This decline in sperm count can again be explained by the testiculotoxic effect of allethrin. Apoptosis causes loss of germ cell layer leading to decreased formation of spermatozoa. Akunna et al (2013) showed a significant reduction in spermatozoa concentration, sperm motility, and normal sperm morphology on exposure to allethrin based mosquito coil when compared to the control groups. Oda and El-Maddawy (2012) found that feeding rats with deltamethrin for 60 days caused a significant reduction in percentage of sperm count and sperm motility. Dahamna et al. (2010) observed decreased sperm count by around 20% in mice following treatment with oral cypermethrin. The reduced sperm count may also be caused by direct effect of pyrethroid on leydig and sertoli cells leading to decrease in testosterone production (Elbetieha et al. 2001). The present study also demonstrates that the effect of allethrin based mosquito coil on sperm count is reversible because after cessation the sperm count of group III did not significantly differ from controls groupie (figure1). Improvement in weight of testis as well as sperm count showed that damage is not permanent and regeneration of germ cell is possible.

In group IV rats, co-administration of Vit. C improved the sperm count. This is again because of the antioxidant activity of Vit. C, it may decrease the oxidative stress in testicular tissue and the damaging effect of free radicals on sperm allowing normal spermatogenesis (Djeffal et al. 2012).

Sperm morphology

More often sperm count may be adequate but still individual may be infertile due to the presence of large number sperms with abnormal morphology. Sperm abnormality assay is a sensitive and reliable endpoint and is widely used to identify germ cell mutagen. Abnormal spermatozoa in mice and rats were reported after exposure to various pyrethroids i.e., fenvalerate and cypermethrin (Bunya and Pati 1990), deltamethrin (Alhazza and Bashandy 1998). The sperm shape abnormalities are caused by genetic damage probably arising from interference by the test substance with genetically controlled differentiation of the cells (Wyrobek et al. 1983). In the present study there was significant increase in the mean percentage of sperm with abnormal morphology in group II (figure 1). Sperms with double head (figure 3), absent head (figure 4) and looped tail (figure 2) was the most common. Other types of abnormality seen were sperm with amorphous head, absent tail, double tail (figure 2-4). The plausible cause behind this study may be due to indirect damage to DNA or defective repair processes or allethrin induced production of ROS leading to gene alteration in germ cell. Nashwa et al. (2008) observed bent tail, coiled tail, distal protoplasmic droplets, detached head and headless sperm on exposing rats to oral deltamethrin. Even after cessation of exposure to mosquito coil in group III, mean percentage of abnormal sperms was high suggesting the genetic damage to be irreversible (Table 1). It has been postulated that vitamin C minimizes testicular cytotoxic effects through preventing the production of the ROS. Such antioxidant action of Vit.C could relieve the germ cells from oxidative damage thereby decreasing the percentage of abnormal sperm. In present study by co-administration with Vit.C, mean percentage of abnormal sperms were decreased significantly which was in concordance with the result of other study (Nashwa et al. 2008).

Conclusion

This study demonstrates the nuisance role of allethrin on male fertility, a common public health problem in developing countries. This study has its own merit that it also depicts that on cessation of exposure for 8 weeks though testicular weight and sperm count improved but not much improvement was seen in sperm with abnormal morphology. It suggest that the either the genetic changes are irreversible or requiring more time to recover. On the other hand Vit. C has a protective role against the toxicity of the allethrin as it worked as an antioxidant.

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Ethical approval

Ethical approval declared from the King George's Medical University Ethics Committee-wide letter No.66/IAH/Pharma-14

Conflict of interest

The authors declare that there is no conflict of interest.

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