Author’s publications
Protective role of Centella asiatica on lead-induced oxidative stress and suppressed reproductive health in male rats


Department of Biotechnology, S.V. University, Tirupati 517 502, India
Department of Zoology, S.V. University, Tirupati 517 502, India

Abstract
Centella asiatica has been mentioned in ancient ayurvedic text of the Indian system of medicine for its properties to promote intelligence. The objective of the present study was to investigate the beneficial effects of C. asiatica on lead-induced oxidative stress and suppressed reproductive performance in male rats. Significant decrease in the weights of testes and epididymis were observed in lead treated animals. Exposure to lead acetate significantly increased malondialdehyde levels with a significant decrease in the superoxide dismutase and catalase activities in the liver, brain, kidneys and testes of rats. Epididymal sperm count, viable sperms, motile sperms and HOS-tail coiled sperms decreased significantly in lead-exposed rats. Testicular steroidogenic enzyme activities also decreased significantly in lead-exposed rats. No significant changes in the selected reproductive variables were observed in the plant extract alone treated rats. Whereas, co-administration of aqueous extracts of C. asiatica to lead exposed rats showed a significant increase in the weights of reproductive organs, reduction in lead-induced oxidative stress in the tissues and improvement in selected reproductive parameters over lead-exposed rats indicating the beneficial role of C. asiatica to counteract lead-induced oxidative stress and to restore the suppressed reproduction in male rats.

© 2011 Elsevier B.V. All rights reserved.

Keywords: Lead Oxidative stress Male reproduction Sperm Centella asiatica and rat

1. Introduction
Infertility is of great concern among the people of reproductive age. It is believed that increase in ‘male-infertility’ observed in wildlife and humans in recent past is an irreversible damage caused by a range of man-made pollutants through different mechanisms (Sharpe, 1993; Becker and Berhane, 1997). Among an array of toxic chemicals, metals are unique environmental toxicants as they tend to possess bioaccumulative, immutable and non-biodegradable properties and pose a serious threat to eco-biological systems (Kakkar and Jaffery, 2005). Lead (Pb) is one of the well-known ubiquitous non-essential metal poisons in the environment. It has been reported that, due to improper management of lead-acid battery recycling operations, many workers who work for their livelihood in lead-based industries and the public and animals are unknowingly exposed to more levels of lead (Benoff et al., 2000; Shaiu et al., 2004). In India, every year approximately eight million lead-acid batteries are produced with consumption of 10,000 kg of lead (Rao et al., 2007). Thus, it is obvious that exposure to lead is implicated in serious health hazards in animals and humans (Hu, 2000). The deterioration of male reproductive health is one of the major manifestations of occupational and/or environmental exposure to Pb toxicity (Kakkar and Jaffery, 2005). Studies of Bonde et al. (2002) and Naha et al. (2003) suggested that men working in Pb based factories showed poor sperm production in terms of quality and density. It has also been reported that workers
exposed to Pb suffered with oligospermia and astenozoospermia (Gennart et al., 1992; Alexander et al., 1996) with altered sperm morphology (Chowdury et al., 1986; Guloik, 1989). In addition, studies of Biswas and Ghosh (2004) demonstrated that lead exposure reduces the activity levels of testicular steroidogenic enzymes in rats.

Although little is known, few studies claim that reproductive toxicity of lead poisoning may be at least in part due to imbalance between pro-oxidant and antioxidant cascades there by generation of more reactive oxygen species (ROS) (Schäfer et al., 2005; Sobeković et al., 2009). Antioxidant enzymes such as superoxide dismutase and catalase play a pivotal role to reduce the oxidative stress. It has been reported that, oxidative stress and a failure of antioxidant defense system causes several sperm abnormalities and result in infertility (Makker et al., 2009). Thus, though unclear, it is supposed that an imbalance between the ROS generation and scavenging system might be one of the reasons for Pb-induced male reproductive toxicity.

Use of herbal extracts has been practiced for centuries in all parts of the world in various systems of medicine like Ayurveda, Siddha, Unani, and Naturopathy. etc. The success of herbal extracts is chiefly because they comprise many positive factors but the factor 'no toxic effects of its own' being important. The use of traditional herbal extracts to combat chemical toxicity is greatly acknowledged in the recent years. Centella asiatica (Indian Pennywort) is a member of Umbelliferae family and popularly known as 'Saraswataku' in southern parts of India, particularly Andhra Pradesh. The medical applications of C. asiatica comprises wound healing property, improvement of memory (Goh et al., 1995) treatment of asthma, renal failures, respiratory problems (Jaganath and Ng, 1999) and other medical uses being treatment of headache and leprosy (Shukla et al., 1999). Few studies also suggest that C. asiatica has ability to counteract arsenic-induced oxidative stress with its antioxidative properties (Flors and Gupta, 2007). Protective effect of C. asiatica on antioxidant tissue defense system against adriamycin-induced cardiomyopathy (Gnanapraksam et al., 2004) and age-related neurological disorders (Subathra et al., 2005) are also well known. Even though several studies suggest the beneficial role of C. asiatica (Zainol et al., 2003), its effects against lead-induced reproductive toxicity is not yet reported. The present study was undertaken to investigate if the oral intake of aqueous plant extract of C. asiatica could modify (a) the oxidative stress and, (b) suppressed reproduction induced by lead in male rats.

2. Materials and methods

2.1. Chemicals

Lead acetate of AR grade was purchased from E-Merck. NADPH, NAD, dehydroepiandrosterone and androstenedione were purchased from Sigma Chemical Company, St Louis, MO. Thiobarbituric acid (purity ≥99%) and malondialdehyde (purity ≥98%) were obtained from Merck, Darmstadt, Germany. All other chemicals used in study were of analytical grade and purchased from local commercial sources.

2.2. Plant materials and preparation of plant extract

C. asiatica (L) used in this study was obtained from the herbal gardens of S.V. Ayurvedic pharmacy, Srinivasa Mangapuram, Tirupati. The taxonomic identification was done with the help of herbarium keeper, Department of Botany, S.V. University, Tirupati. The whole plant was washed with sterile distilled water, cleaned and shade dried at room temperature in sterile conditions. The whole plant was pulverized using a mechanical grinder to a fine powder. The extract was prepared according to the method described earlier (Nishnu Rao et al., 1996).

2.3. Animals and maintenance

Adult male albino rats (70-80 days old) of Wistar/NIN (WNN) strain were used for this study. The rats were procured from an authorized vendor (M/S Raghavendra Enterprises, Bangalore, India) and were housed (three per cage) in polypropylene cages (47 cm x 34 cm x 20 cm) containing sterile paddy husk (procured locally) as bedding material and maintained at 22-25°C under a well regulated light and dark (12:12 h) schedule at the Animal Facility of the Department of Zoology, Sri Venkateswara University. The rats were fed on standard rat Chow (HLL Animal feed, Bangalore, India) and water ad libitum. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (vide no. IAC/CPCSEA/638/01/a/CPCSEA).

2.4. Experimental design

The animals were equally randomized to four groups (one control group and three treatment groups), each group consisting of six rats. The rats in group I served as control and was allowed ad libitum access to tap water. The animals in groups II were allowed ad libitum access to tap water containing 819 mg/L lead (0.15% lead acetate) for 70 days. A stock solution of 1% dissolved in distilled water; immediately prior use, it was diluted with filtered tap water to the desired concentration. Rats in group III were given orally aqueous extracts of C. asiatica (200 mg/kg body weight/day) through intragastric feeding tube over a period of 70 days and the animals in group IV received same experimental regimen as that of group II and in addition, the aqueous extracts of C. asiatica was also given orally for a period of 70 days. The doses for lead and C. asiatica were selected on the basis of previously published studies (Ronis et al., 1996; Kumar and Gupta, 2002; Sharma and Sharma, 2002).

2.5. Necropsy

The body weight of rats was recorded at the time of initiation and completion of the experiment (prior to necropsy). Prior to the termination of the experiment, rats were fasted over night, weighed and killed by using overdose of anesthetic ether on the day following the last treatment. Liver, brain, kidney, testis, epididymis, prostate gland, via deference and seminal
vesicle were quickly removed, cleared of the adhesive tissues and weighed wet to the nearest milligram and used for the determination of tissue somatic indices (TSI).

\[ TSI = \frac{\text{weight of the tissue (g)}}{\text{body weight of the animal (g)}} \times 100 \]

2.6. Analysis of sperm parameters

Epididymal sperms were obtained by chopping cauda epididymis in 5.0 ml of Ham's F12 medium. The sperms were counted by using a Neubauer Chamber, as describe by Belsey et al. (1980). Progressive sperm motility was evaluated by the method as described previously (Belsey et al., 1980), within 5 min following their isolation from cauda epididymis at 37 °C and the data were expressed as percent motility. The viability of the sperm was determined using 1% tryphan blue reagent (Tisbor and Chacon, 1981). The function (HOS coil ing) of the sperm was determined by exposing the sperms to hyposmotic solution and observed for coiled tails under the microscope and the percent of coiling was estimated using the method described by Jeyendran et al. (1992).

2.7. Assay of testicular steroidogenic enzymes

The testicular tissue was homogenized in ice-cold Tris-HCl buffer (pH 8.8). The microsomal fraction was separated and used as enzyme source. The activities of 3p hydroxysteroid dehydrogenase (3p HSD) (EC 1.1.1.51) and 17p hydroxysteroid dehydrogenase (17p HSD) (EC 1.1.1.64) were determined by the method of Bergmayer (1974). The enzyme assays were made under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration.

2.8. Estimation of lipid peroxidation and assay of antioxidant enzymes in testes

Liver, brain, kidneys and testes were isolated, cleared from the adhering tissues and different subcellular fractions were prepared for the estimation of lipid peroxidation products and also to determine the activity levels of antioxidant enzymes such as superoxide dismutase and catalase.

2.8.1. Sub-cellular fractionation of tissues

Different sub-cellular fractions were obtained by the differential centrifugation method. Briefly, a 10% (W/V) homogenate was prepared in ice-cold sucrose solution with the help of a motor-driven glass Teflon homogenizer. The homogenate was centrifuged at 1000 g for 10 min at 4 °C to obtain the nuclear pellet. The microsomal pellet was prepared by the calcium chloride (CaCl2) sedimentation method of Kamath and Narayan (1972).

2.8.2. Lipid peroxidation

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance was measured by the method of Ohkawa et al. (1979).

2.8.3. Assay of antioxidant enzymes

Superoxide dismutase (EC 1.15.1.1) (SOD) was assayed in the microsomal fraction according to its ability to inhibit the autooxidation of epinephrine at alkaline medium (Mishra and Fridovich, 1972). The activity of SOD was expressed in units per min per mg protein. Catalase (EC 1.11.1.6) activity was determined based on its ability to decompose H2O2 (Chance and Machly, 1955). The activity of enzyme was expressed in μmol of hydrogen peroxide consumed per min per mg protein. Protein content in the enzyme source was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.9. Scanning electron microscope (SEM) analysis of sperms

The details of SEM analysis were essentially similar to the procedure described earlier (Lohiya et al., 2002). In brief, spermatozoa were washed with phosphate buffer (0.01 mol/l, pH 7.2) and pelleted by centrifugation. The sperm pellets were fixed in 2.5% glutaraldehyde for 30 min and washed thrice in phosphate buffer followed by distilled water. A thin film of spermatozoa was smeared on a clean glass slide, air dried, then mounted on SEM stub, sputtered with gold panicles, and observed under SEM (EVO MA 15).

2.10. Statistical analysis

The data were statistically analyzed using analysis of variance (one way ANOVA) followed by Dunnet's test. Differences were considered to be significant at p < 0.01. The data were presented as mean ± S.D. All statistical tests were performed using Statistical Package for Social Sciences (SPSS Inc., Chert- sney, UK).

3. Results

3.1. General toxicity

The rats were observed for responses with respect to overall appearance, body position, activity, co-ordination or gait, and behavior. No significant changes in lacrimation, urination, respiration, vocalization, postural or gait abnormalities were observed in any of the control and treated rats. All the animals were apparently normal and no unusual behaviors (viz., head flicking, head searching, biting, licking, self-mutilation, circling, and walking back-wards) were observed in any of the rats. However, the animals exposed to lead acetate alone showed slight sluggishness in the last week.

3.2. Body and organ weights

Body weight was not found to change in any of the treated groups (Table 1). No significant changes in the relative weights of liver, brain and kidney were observed in control and experimental rats (Table 1). The indices of testis and epididymis were significantly decreased in lead treated rats over control animals. No significant changes in the testis and epididymis indices were observed in CA alone treated rats when compared with control rats (Table 1). Significant increase in the
weights of testis and epididymis were observed in rats co-administered with CA when compared with Pb treated rats.

3.3. Sperm analysis

The effect of lead and co-administration of CA on total epididymal sperm count, sperm motility and sperm viability is shown in Table 2. No significant changes in sperm parameters were observed in rats after CA administration. On the other hand, significant decrease was observed in epididymal sperm count, sperm viability, sperm motility and also percentage of number of HOS-tail coiled sperms in rats exposed to lead for 70 days. The sperm from control rat was observed under scanning electron microscope and the sperm was with hook-shaped head (Fig. 1A). No morphological abnormalities were observed in sperms of control rats whereas several (45%) sperms were observed with misshapen head (pear shaped, banana head, pin head, mutilated head and headless) after exposure to lead (Fig. 1B). Midpiece and tail of the sperm was normal in rats exposed to lead. Co-administration of CA treatment significantly increased the selected sperm variables in lead acetate exposed rats when compared with Pb treated rats (Table 2). All the sperms in the rats co-administered with CA are apparently normal in shape.

3.4. Steroidogenic pathway enzymes in testes

Table 3 shows the activity levels of testicular 3β- and 17β-hydroxysteroid dehydrogenase in control and experimental rats. Significant decrease in the testicular 3β- and 17β HSD activities were observed in Pb exposed rats as compared to control rats. No effect of CA on these enzymes was noted when administered to normal animals. On the other hand, co-administration of CA with lead had significantly increased both these enzymes when compared with Pb treated rats but still lower than the control levels (Table 3).

3.5. Antioxidant enzymes and lipid peroxidation

The activity levels of SOD and catalase were significantly decreased in the microsomal fraction of liver, brain, kidney and testis of rats exposed to lead with a significant increase in the lipid peroxides (Table 4) as compared to control rats. The activity levels of these enzymes were not significantly altered on CA treatment (Table 4). Significant elevation in the activity levels of SOD and catalase were observed with significantly reduced levels of lipid peroxidation products in lead exposed rats co-administered with CA as compared to lead exposed rats (Table 4).

4. Discussion

There is an increasing concern that environmental contaminants disrupt male reproduction of wildlife and humans and play an important role in decline of quality and density of human semen (Sharpe, 2000). The present study was carried out to evaluate the beneficial effect of C. asiatica extract on lead induced reproductive toxicity in Wistar rats. C. asiatica has been mentioned in ancient Ayurvedic text of the Indian system of medicine for its properties to promote intelligence (Chopra et al., 1956). It has also been reported to possess anti-ulcer
Table 2 - Lead-induced changes in sperm parameters and their response to administration of aqueous extract of Centella asiatica (CA) in rats during 70 days of exposure.

<table>
<thead>
<tr>
<th>Sperm parameters</th>
<th>Control</th>
<th>Experimental groups</th>
<th>Pb</th>
<th>CA</th>
<th>Pb + CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (millions/ml)</td>
<td>64.28 ± 4.38</td>
<td>47.65 ± 3.88</td>
<td>66.18 ± 6.67</td>
<td>57.34 ± 3.89</td>
<td></td>
</tr>
<tr>
<td>Visible sperm (%)</td>
<td>71.23 ± 5.61</td>
<td>49.22 ± 6.19</td>
<td>70.96 ± 4.18</td>
<td>66.58 ± 4.09</td>
<td></td>
</tr>
<tr>
<td>Motile sperm (%)</td>
<td>65.70 ± 3.84</td>
<td>42.89 ± 4.69</td>
<td>66.09 ± 3.89</td>
<td>61.72 ± 5.76</td>
<td></td>
</tr>
<tr>
<td>HOS tail coiled sperm (%)</td>
<td>60.84 ± 4.17</td>
<td>38.57 ± 5.56</td>
<td>59.88 ± 3.98</td>
<td>57.89 ± 4.12</td>
<td></td>
</tr>
<tr>
<td>Abnormal sperm (%)</td>
<td>1.0</td>
<td>45.0</td>
<td>1.0</td>
<td>4.67</td>
<td></td>
</tr>
<tr>
<td>Pear head sperms</td>
<td>3/300</td>
<td>24/300</td>
<td>2/300</td>
<td>2/300</td>
<td></td>
</tr>
<tr>
<td>Banana head sperms</td>
<td>6/300</td>
<td>5/300</td>
<td>6/300</td>
<td>2/300</td>
<td></td>
</tr>
<tr>
<td>Headless sperms</td>
<td>11/300</td>
<td>1/300</td>
<td>1/300</td>
<td>2/300</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 6 individuals. Values in the parentheses are percent change from that of control. Mean values with same superscripts do not differ significantly from each other, p < 0.01.

Table 3 - Lead-induced changes in testicular 3a- and 17α-hydroxysteroid dehydrogenase activity levels and their response to administration of aqueous extract of Centella asiatica (CA) in rats during 70 days of exposure.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>Experimental groups</th>
<th>Pb</th>
<th>CA</th>
<th>Pb + CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α-Hydroxysteroid dehydrogenase (nmol of NAD converted to NADH/mg protein/min)</td>
<td>18.23 ± 2.46</td>
<td>7.13 ± 3.35</td>
<td>18.72 ± 1.43</td>
<td>13.78 ± 2.54</td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxysteroid dehydrogenase (nmol of NADPH converted to NADH/mg protein/min)</td>
<td>9.26 ± 1.95</td>
<td>4.91 ± 1.25</td>
<td>10.11 ± 1.14</td>
<td>9.03 ± 0.91</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 6 individuals. Values in the parentheses are percent change from that of control. Mean values with same superscripts do not differ significantly from each other, p < 0.01.
balance. In the present study, the sperm endpoints such as epididymal sperm count, sperm motility, sperm viability were significantly decreased in lead treated rats. Further, the decrease in the percent number of tail coiled sperms as evidenced by hypo-osmotic swelling test suggests that membrane integrity of the sperms is deteriorated. Lead exposure also results in severe morphological abnormalities in the head of sperms. Testicular 3β- and 17β-hydroxysteroid dehydrogenases play an important role in the biosynthesis of testosterone (Hinshelwood et al., 1994), which is pre-requisite for the sperm production and maturation. Significant decrease in the activity levels of these enzymes was indicative of reduced steroidogenesis in Pb treated rats. This suggests that Pb induced oxidative stress might affect Leydig cells and eventually disrupts cascade of steroidogenic pathway. This in turn results in decreased synthesis of male hormone thereby affects the sperm production (Hlamadouche et al., 2009).

The deterioration in the selected sperm parameters might be due to increased oxidative stress during Pb intoxication. Sperm plasma membrane, being rich in polyunsaturated fatty acids (PUFA), is highly susceptible to reactive oxygen species (ROS) attack. The results are in consonance with earlier reports of Berry et al. (2002) and Jensen et al. (2006). Poor sperm quality caused by oxidative stress due to generation of ROS has been reported to result in infertility (Aitken and Baker, 2006). Several studies suggest the correlation between increased ROS production and decreased sperm motility (Agarwal et al., 1994; Armstrong et al., 1999). The mechanism of ROS induced altered sperm motility is still unclear. However, it is hypothesized that H$_2$O$_2$ one of the lipid peroxidation products, might diffuse across the membrane and affect the vital enzymes in the sperms (Makker et al., 2009) thereby results in decreased sperm motility.

It is evident that Pb crosses blood-testis barrier (Dixon Robert, 1986) and alters testicular functions like production
of sperms and testosterone. In the present study, Pb toxicity caused testicular oxidative stress by increasing the levels of lipid peroxidation and decreasing the activities of SOD and catalase in testes. In general, SOD is the first line of defense against oxidative stress (Hassan and Schellhorn, 1988) and play a pivotal role in dismutation of superoxide anions to hydrogen peroxide and catalase neutralizes hydrogen peroxides to molecular oxygen and water (Inal et al., 2001). The decrease in these enzymes in Pb-treated rats clearly postulates improper dismutation of superoxides and improper decomposition of H$_2$O$_2$. Studies of Marchlewicz et al. (2007) reported increased lipid peroxidation products in the testes and epididymis of rats after exposure to lead. Hsu and Guo (2002) demonstrated that Pb exposure causes generation of ROS and alterations in the antioxidant defense systems in animals and occupationally exposed workers.

In the present study, CA treatment was found to significantly decrease the levels of lipid peroxidation and increase the activities of superoxide dismutase and catalase in the testes as compared to control rats. Epididymal sperm count, sperm viability and motility were also significantly increased in the rats subjected to co-administration of CA extract as compared to lead-exposed rats. These findings indicate the possible role of CA, characterized by increased level of antioxidant enzymes and decreased lipid peroxidation in testis, in combating the testicular toxicity of lead. The antioxidant potential of CA has been reported earlier by several workers (Zainol et al., 2003; Gupta and Flora, 2006). CA contains high amounts of potent antioxidant compounds, such as madecassic acid, asiatic acid and asiaticoside (Inamdar et al., 1996; Shukla et al., 1999; Khare, 2004). Besides, the concentrations of phenolic compounds are more in CA (3.23-11.7 g/100 g dry sample) and these compounds are the major contributors to antioxidant activities of CA (Zainol et al., 2003). They also identified a strong correlation ($r^2 = 0.90$) between antioxidant activities and phenolic levels, suggesting that phenolic compounds are
probably responsible for the antioxidative activities of C. asiasi.
A similar finding was reported by Gardner et al. (2000),
who suggested that phenolic compounds are the major con-
tributors to the antioxidative activity of apple, pineapple and
vegetable juices. Although phenolic compounds are found to
be the major contributors to the antioxidative activity in C. asi-
siasi, the identity of these compounds still remains unknown.

5. Conclusions

It could be concluded from the present results that co-
administration of aqueous extract of C. asiasi offers
significant protection to lead-induced oxidative stress by
decreasing lipid peroxidation and activating antioxid-
antly enzymes in the testes, thereby ameliorating lead-induced
repressed reproduction in male rats. From the study, however, it
is not clear whether CA has ability to chelate lead and thereby
reduces oxidative injury. It will be interesting to evaluate the
reproductive performance of rats exposed to Pb and Pb - CA.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors are grateful to the Head, Department of Biotech-
nology, as well as Head, Department of Zoology, S.V. University,
Tirupati for providing the laboratory and animal facilities. One
of the authors (PSR) is thankful to University Grant Commis-
sion, New Delhi [F.4(2)-2006(SR)] and Indian Council of
Medical Research, New Delhi for providing financial assistance
(5/10/5/2005-RHII). We thank Prof. K.V.S. Sarma, Department
of Statistics, S.V. University for the statistical analysis of data, Mr.
K. Sivakumar Reddy, Department of physics, S.V. University for
SEM analysis and Mr. S. Umasankar, Department of Zoology,
S.V. University for maintaining rat colony. The authors declare
that the experiments conducted during these studies comply with
the current laws of their country.

REFERENCES

spem parameters with levels of reactive oxygen species in
Alessander, B.H., Checkoway, H., van Netten, C., Muller, C.H.,
Ewers, T.G., Kaufman, J.D., Mueller, B.A., Vaughan, T.L.,
Armstrong, J.S., Rajasekaran, M., Chamulikitran, W., Gotri, P.,
oxygen species induced effects on human spermatozoa
movement and energy metabolism. Free Radic. Biol. Med. 26,
859–880.
Belezy, M.A., Moghissi, K.S., Ellassion, R., Paulsen, C.A., Callegos,
Examination of Human Semen and Semen Cervical Mucus
Interaction. Press concern, Singapore.
environmental exposure to lead and cadmium. Hum. Reprod.
Update 6, 107–121.
Enzymatic Analysis. Verlag Chemie/Academic Press,
Weinheim/New York, pp. 467–489.
Berry Jr, W.D., Mortier, C.M., Lau, Y.S., 2002. Lead attenuation of
episodic growth hormone secretion in male rats. Int. J.
Toxicol. 21, 93–98.
Bonde, J.P., Joffe, M., Apostoli, P., Dal, A., Kiesz, P., Spomo, M.,
Caruso, F., Giwerman, A., Bisanti, L., Forg, S., Vanhoorne, M.,
Comhei, F., Zachowie, W., 2002. Sperm count and
chromatin structure in men exposed to inorganic lead: lowest
Medicinal Plants. CSIR, New Delhi, p 58.
Chowdhury, A.K., Chiniy, N.J., Gautam, A.K., Rao, R.V., Parikh, D.J.,
Indian J. Pharmacol. 35, 257–274.
system. In: Klaassen, C.D. (Ed.), Casarett and Doull's
433–447.
aqueous extract against arsenic-induced oxidative stress and
The relative contributions of vitamin C, carotenoids and phenolics
to the antioxidant potential of fruit juices. Food Chem. 68,
471–474.
Gennart, J.F., Bernard, A., Lauwevres, R., 1992. Assessment of
thyroid, testes, kidney and autonomic nervous system
Health 64, 46–57.
Gnenprapagunan, A., Kumar Ebenzer, K., Satish, V., Govindaraju,
on antioxidant tissue defense system against adriamycin
induced cardiomyopathy in rats. Life Sci. 76, 585–597.
Medical Plants for the Treatment of Cardiovascular
Guilok, M.E., 1989. Spermogenesis and maturation of
55, 73–87.
Gupta, R., Flora, S.J.S., 2006. Effect of Centella asiatica on arsenic
induced oxidative stress and metal distribution in rats. J.
Appl. Toxicol. 26, 213–222.
Reproductive toxicity of lead acetate in adult male rats. Am. J.
Hsu, P.C., Guo, Y.L., 2002. Antioxidant nutrients and lead toxicity.
Toxcoloy 180, 33–44.
Hassan, H.M., Scheihorn, H.E., 1988. Superoxide dismutase an
antioxidant defense enzyme. In: Gerutti, P.A., Fridovich, I.,


This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright
Toxicology

Protective effects of N-acetylcysteine against arsenic-induced oxidative stress and reprotoxicity in male mice

P. Sreenivasula Reddy a, b, *, G. Pushpa Rani a, S.B. Sainath a, R. Meena a, Ch. Supriya a

a Department of Biotechnology, Sri Venkateswara University, Tirupati 517 502, India
b Department of Zoology, Sri Venkateswara University, Tirupati 517 502, India

A R T I C L E   I N F O

Article history:
Received 17 November 2010
Accepted 22 August 2011

Keywords:
Sodium arsenite
N-Acetylcysteine
Lipid peroxidation
Sperm
Antioxidant enzymes
Male mice

A B S T R A C T

Arsenic is a well-known environmental toxic metalloid element and carcinogen that affects multiple organ systems including tissue lipid peroxidation and reproduction. The present study was aimed to investigate the protective role of N-acetylcysteine (NAC) on arsenic-induced testicular oxidative damage and antioxidant and steroidogenic enzymes and sperm parameters in mice. Arsenic was administered through drinking water to mice at a concentration of 4.0 ppm sodium arsenite (actual concentration 2.3 ppm arsenic) for 35 days. The body weight of treated mice did not show significant change as compared with the control mice. In arsenic exposed mice there was a significant decrease in the weight of the testis, epididymis and prostate gland as compared with the control animals. Significant reduction was observed in epididymal sperm count, motile sperms and viable sperms in mice exposed to arsenic indicate decreased spermatogenesis and poor sperm quality. The activity levels of testicular 3β- and 17β-hydroxysteroid dehydrogenases and circulating levels of testosterone were also decreased in arsenic exposed mice indicating reduced steroidogenesis. A significant increase in the activities of lipid peroxidation and a significant decrease in the activities of antioxidant enzymes were observed in the testis of mice exposed to arsenic. In addition, significant increase in the testicular arsenic levels was observed during arsenic intoxication. No significant changes in the oxidation status and selected reproductive variables were observed in the N-acetylcysteine alone treated mice. Whereas, intra-peritoneal injection of NAC to arsenic exposed mice showed a significant increase in the weights of reproductive organs, reduction in arsenic-induced oxidative stress in the tissues and improvement in steroidogenesis over arsenic-exposed mice indicating the beneficial role of N-acetylcysteine to counteract arsenic-induced oxidative stress and to restore the suppressed reproduction in male mice.

© 2011 Elsevier GmbH. All rights reserved.

Introduction

Arsenic is a well-known environmental toxic metalloid element and well known as human carcinogen [1]. Anthropogenic emissions of arsenic and its compounds into the environment occur mainly due to extensive use of arsenic in leather pigments, antifungal wood preservatives, copper smelting, pesticides and herbicides, other mining industries and in medical applications to treat leukemia [2,3]. It has been suggested that acute and chronic exposure to arsenic causes several detrimental effects on human health [4]. Acute problems of arsenic include nausea, vomiting, abdominal pain, encephalopathy and neuropathy, whereas chronic exposure to arsenic comprises of a range of cancers such as skin, lung, bladder and liver and metabolic disorders like diabetes, gastrointestinal tract problems, heart diseases and neuronal problems [5]. Although many studies reported the toxic effects of arsenic, the data related to the arsenic induced-reproductive toxicity is limited. Arsenic is acknowledged as a reproductive toxicant in humans [6] and coupled with prostate cancer [7]. Studies in animal models demonstrated that arsenic exposure affects the growth of reproductive organs [8,9]. Effects on the male reproductive system including impaired reproductive capacity, inhibition of spermatogenesis [10], reduced steroidogenesis [11] and atrophic changes of the testis [8] have been documented in arsenic exposed animals. Arsenic intoxication has been reported to cause degeneration of spermatogenic and Leydig cells [8]. Previous studies also suggested that exposure of males to arsenic affects the weights of testis and accessory sex organs, accompanied by decreased androgen levels [12]. In addition, arsenic acts as an endocrine disruptor and thereby alters the pituitary-testicular axis and affects testosterone levels in experimental animals studied [9]. Recent studies also demonstrated that arsenic exposure modulates the estrogen levels leading to preneoplastic lesions in the testis of experimental animals [13]. Studies of Jana et al. [14] also hypothesized that arsenic at least in part mediates estrogen and interferes with hypothalamo-pituitary-testicular
axis in adult male rats. Thus, it is evident that arsenic affects all most all compartments and physiology of male reproductive tract. However, the plausible mechanism of action of arsenic-induced suppressed reproductive health is still unclear. Several mechanisms have been put forth to decipher the possible mode of action of arsenic toxicity. Many studies claim that arsenic poisoning is associated with oxidative stress, a condition exists due to an imbalance between pro- and antioxidant status [15-17].

It is well known that arsenic-induced acute and chronic toxicities are largely dependent on its chemical form and physical state. The trivalent nature of arsenic is considered most toxic when compared to pentavalent form of arsenic. Being an electrophilic agent, arsenic binds to sulfhydryl groups on proteins and modulates protein metabolism and/or indirectly by generation of reactive oxygen species (ROS) which in turn will affect vital molecules of the cells [18-20]. In general, to overcome the ROS attack, animal cells are equipped with an intrinsic scavenging system with enzymes and antioxidant molecules [21]. Thus, a failure in the neutralizing system against ROS adversely affects the organs and their processes. Recently, it has been reported that alterations in the activity levels of antioxidant enzymes and increase in the lipid peroxidation products causes hepatotoxicity and nephrotoxicity in arsenic exposed rats [22]. Studies of Chang et al. [23] demonstrated that arsenic-mediated reproductive toxicity is mainly due to depleted levels of testicular glutathione content with increased malondialdehyde levels. It seems apparent that arsenic toxicity, at least in part, originates from oxidative stress and thus, a therapeutic strategy to increase the antioxidant capacity of cells against arsenic poisoning is considered important. This may be accomplished by either removal/prevention of arsenic from the tissues and interactions with cellular macromolecules, respectively, or by provoking the cellular antioxidant defenses through endogenous supplementation of antioxidant molecules. Among those strategies, chelating therapy is widely used to treat arsenic poisoning. The chelating agents used to prevent arsenic accumulation in the soft tissues include vitamins [24], thiol compounds [25] and micronutrients [26], as these factors have been known to displace toxic metals from the tissues and are involved in the modulation of various biochemical aspects.

N-Acetylcysteine (NAC), the thiol based antioxidant, plays an important role in the protection of cellular constituents against oxidative damage and in the detoxification of many electrophiles. The hypothetical action of NAC originates from its ability to stimulate and to sustain the intracellular levels of reduced glutathione levels and to detoxify ROS. The other advantage of NAC is its efficiency to chelate toxic metals. Earlier studies of Banner et al. [27] reported that NAC plays a vital role in the chelation of chromium, lead and boron. Recently, studies of Modi et al. [25] reported that in co-supplementation of NAC along with zinc mitigates the arsenic-induced oxidative stress in liver and kidney of male rats. Considering the facts that (a) arsenic affects many vital parts of reproductive system including testis, (b) one of the possible mechanisms of action of arsenic is by altering pro- and anti-oxidant status and (c) NAC is a non-natural precursor of reduced glutathione (GSH) and is implicated in reducing the risk of arsenic toxicity in vital parts, the present study was aimed to investigate the effects of supplementation of NAC on arsenic-induced testicular oxidative stress and suppressed male reproductive health in mice.

Materials and methods

Chemicals

Sodium arsenite (NaAsO₂) was obtained from S.D. Fine Chemicals, Mumbai, India and N-acetylcysteine, androstenedione, dehydroepiandrosterone, NAD and NADPH were purchased from Sigma Chemical Company, St. Louis, MO. Thiobarbituric acid was obtained from Merck-Chuchardt, Germany. All other chemicals were of analytical grade and obtained from local commercial sources.

Animals

Male Swiss albino mice (60-70 days age with a body weight 28 ± 3g) were procured from an authorized vendor (M/S Raghavendra Enterprises, Bangalore, India) and were housed (four per cage) in polypropylene cages (18 in. × 10 in. × 8 in.) lined with sterile padded husk, under standard laboratory conditions (temperature 30 ± 2°C; light and dark 12:12 h) at the Animal Facility of the Department of Zoology, Sri Venkateswara University. The mice were fed on standard pellet chew (purchased from HIL Animal Feed, Bangalore, India) and water ad libitum. All animal procedures were carried out in accordance with the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals. This experiment was reviewed and approved by the Institutional Animal Care and Ethics Committee at S.V. University (418/01A/CPSEA) and all procedures were in compliance with the laws of the country.

Experimental design

In the present investigation, mice were equally randomized to four groups (one control group and three treatment groups), each consisting of eight animals. The mice in group I served as control and was allowed ad libitum access to tap water. The animals in group II were allowed ad libitum access to tap water containing 4.0 ppm sodium arsenite (actual concentration, 2.3 ppm arsenic) instead of normal water and maintained for a period of 35 days. A stock solution of 100 ppm arsenic was prepared in distilled water, immediately prior use, it was diluted with filtered tap water to the desired concentration. Mice in group III were injected intraperitoneally 75 mg/kg body wt. N-Acetylcysteine for 5 alternate days and maintained on tap water for 35 days and animals in group IV received same experimental regimen as that of group II and in addition, NAC was also injected on 2nd, 4th, 6th, 8th and 10th day of arsenic exposure. The arsenic dose selected for the present study is based on the levels of arsenic in drinking water in several areas of India and other countries, where this trace element is present in range above the permissible limit (0.01 ppm)[28,29]. In the present study, the animals were exposed to arsenic at a concentration below no observed effect level (NOEL) in order to evaluate if low concentrations of arsenic induce oxidative stress in tissues and suppress the reproduction in male mice. Further, the dose selection (4.0 ppm sodium arsenite) was also based on the dose–response kinetic studies by evaluating the malondialdehyde levels in the testis of mice (authors’ unpublished data). The dose of NAC was selected on the basis of previously published reports suggesting that NAC were not toxic to humans or animals at this dose [30].

Necropsy

After completion of the experimental period (35 days), the mice were fasted overnight, weighed and killed by using overdose of anesthetic ether. Blood samples from the control and treatment mice were collected by cardiac puncture. Blood was allowed to clot at room temperature and was centrifuged at 2000 × g for 10 min to separate serum. The serum samples were stored at -20°C in microfuge tubes and were used for analyzing the level of hormones. Testes, epididymids, seminal vesicles, vas deferens and ventral prostate were weighed after clearing the adhering tissues using Shimadzu electronic balance (model No: BL-220H), to
the nearest milligram. Epididymal fluid was diluted to a volume of 2.5 mL with 1.15% potassium chloride solution. To 2.5 mL of homogenate, 0.5 mL of saline (0.9% sodium chloride), 1.0 mL of (20% W/V) Trichloroacetic acid (TCA) was added. The contents were centrifuged for 20 min on a refrigerated centrifuge at 4000 x g. To 1.0 mL of supernatant, 0.25 mL of TBA reagent was added and the contents were incubated at 55 °C for 1 h. One milliliter of n-butanol was added to it. After thorough mixing, the contents were centrifuged for 15 min at 4000 x g in a refrigerated centrifuge. The organic layer was transferred into a clear tube and its absorbance was measured at 532 nm. A standard curve was constructed extrapolating the amount to the measured absorbance. The rate of lipid peroxidation was expressed as μmoles of malondialdehyde formed/g wet wt. of tissue.

Determination of protein content in the enzyme source

The protein content in the enzyme source was estimated by the Lowry et al. [40] method using bovine serum albumin as standard.

Determination of arsenic levels in testis

Arsenic levels in testes were estimated according to the method of Ballentine and Burford [41]. To 100 mg of tissue, 1 mL of concentrated nitric acid was added, followed by 1 mL of perchloric acid. The sample was then digested over a sand bath until the solution became clear and yellow in color. If the color of the digest was brown, more nitric acid and perchloric acid were added and the digestion was repeated. The digest was made up to the known volume with deionized water. Aliquots of this were used to estimate arsenic by using atomic absorption spectrophotometer (Perkin-Elmer model No. 2380).

Determination of serum testosterone levels

Serum level of testosterone was determined by enzyme linked immunosorbant assay (ELISA) using kits from Diagnostic System Laboratories (DSL), Inc., Webster, Texas, USA. The assay was done strictly according to the procedure given along with the kit. The sensitivity of the assay was calculated as 0.002 ng and intraassay variation was 5%. All of the samples were run at the same time to avoid interassay variation. The serum level of testosterone was expressed as ng/mL.

Statistical analysis

The data were statistically analyzed using analysis of variance (one-way ANOVA) followed by Dunnett's test. Differences were considered to be significant at p < 0.01. The data were presented as

Antioxidant enzymes

Testicular microsomal fractions were obtained by the differential centrifugation method according to the method described by Chainy et al. [36]. Superoxide dismutase (EC 1.15.1.1) (SOD) was assayed in the microsomal fraction according to its ability to inhibit the autoxidation of epinephrine at alkaline medium [37]. The activity of SOD was expressed in units per mg protein. Catalase (EC 1.11.1.6) activity was determined based on its ability to decompose H2O2 [38]. The activity of enzyme was expressed in μmol of hydrogen peroxide consumed per min per mg protein.

Determination of lipid peroxidation

The level of lipid peroxidation in the tissues was measured in terms of malondialdehyde (MDA; a broken down product of lipid peroxidation) content and determined by using the thiobarbituric acid (TBA) reagent. The reactivity of TBA is determined with minor modifications of the method adopted by Hiroshi et al. [39]. The testes were homogenized (10%, W/V in 1.15% potassium chloride solution. To 2.5 mL of homogenate, 0.5 mL of saline (0.9% sodium chloride), 1.0 mL of (20% W/V) Trichloroacetic acid (TCA) was added. The contents were centrifuged for 20 min on a refrigerated centrifuge at 4000 x g. To 1.0 mL of supernatant, 0.25 mL of TBA reagent was added and the contents were incubated at 55 °C for 1 h. One milliliter of n-butanol was added to it. After thorough mixing, the contents were centrifuged for 15 min at 4000 x g in a refrigerated centrifuge. The organic layer was transferred into a clear tube and its absorbance was measured at 532 nm. A standard curve was constructed extrapolating the amount to the measured absorbance. The rate of lipid peroxidation was expressed as μmoles of malondialdehyde formed/g wet wt. of tissue.

Determination of protein content in the enzyme source

The protein content in the enzyme source was estimated by the Lowry et al. [40] method using bovine serum albumin as standard.

Determination of arsenic levels in testis

Arsenic levels in testes were estimated according to the method of Ballentine and Burford [41]. To 100 mg of tissue, 1 mL of concentrated nitric acid was added, followed by 1 mL of perchloric acid. The sample was then digested over a sand bath until the solution became clear and yellow in color. If the color of the digest was brown, more nitric acid and perchloric acid were added and the digestion was repeated. The digest was made up to the known volume with deionized water. Aliquots of this were used to estimate arsenic by using atomic absorption spectrophotometer (Perkin-Elmer model No. 2380).

Determination of serum testosterone levels

Serum level of testosterone was determined by enzyme linked immunosorbant assay (ELISA) using kits from Diagnostic System Laboratories (DSL), Inc., Webster, Texas, USA. The assay was done strictly according to the procedure given along with the kit. The sensitivity of the assay was calculated as 0.002 ng and intraassay variation was 5%. All of the samples were run at the same time to avoid interassay variation. The serum level of testosterone was expressed as ng/mL.

Statistical analysis

The data were statistically analyzed using analysis of variance (one-way ANOVA) followed by Dunnett's test. Differences were considered to be significant at p < 0.01. The data were presented as
Results

The animals in arsenic-exposed, NAC-injected and arsenic + NAC treated groups did not show any clinical signs of toxicity, abnormal behaviour and none died during the 35 days of experimental period. There was no significant change in food and water intake in treated groups, when compared with control mice (data not shown). Body weights of mice at the end of the experiment (35 days) were not found to change in any of the treatment groups (Table 1). A significant decrease in the weight of testis (–25.71%), epididymides (–17.39%) and prostate glands (–28.37%) was observed in mice exposed to arsenic compared to control mice. No significant changes in the tissue indices were observed in NAC alone treated mice when compared with control mice (Table 1). However, NAC given to arsenic exposed mice resulted in a significant increase in the weight of testis, epididymis, seminal vesicles and vestril prostate as compared to arsenic exposed mice (Table 1).

Exposure to 4.0 ppm sodium arsenite for 35 days resulted in significant accumulation of arsenic in testes of mice (200.00%) over control mice, whereas supplementation of NAC diminished the arsenic content remarkably in the testis of arsenic exposed mice (–34.72%) when compared with arsenic treated mice (Fig. 1). However, the level of arsenic in NAC treated mice is significantly higher than control. No significant changes were observed in arsenic content in testis of mice after NAC alone administration (Fig. 1).

The effect of arsenic and co-administration of NAC on total epididymal sperm count, sperm motility and sperm viability is observed in mice after NAC alone administration. On the other hand, co-administration of NAC with arsenic had significantly increased both these enzymes (3β-HSD and 17β-HSD: 60.42% and 68.03%, respectively) when compared with arsenic alone treated mice but still lower than the control levels (Table 3). Serum level of testosterone was found to decrease significantly in mice exposed to arsenic as compared to control mice (Table 3). In mice subjected to both arsenic exposure and NAC, a significant increase in level of serum testosterone occurred as compared to mice treated with arsenic alone (Table 3). No significant change in serum testosterone level was observed in mice injected with NAC alone.

In mice exposed to arsenic, a significant increase was observed in the level of lipid peroxidation in the testis as compared to control mice (Table 4). Interestingly, NAC treatment significantly decreased MDA levels in testis of arsenic exposed mice compared to mice treated with arsenic alone. The MDA levels were not significantly altered on NAC treatment (Table 4). The activity of antioxidant enzymes like superoxide dismutase and catalase were significantly decreased in the testis of mice exposed to arsenic. Administration of NAC to arsenic exposed mice significantly increased the activities

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>NAC</th>
<th>As</th>
<th>As+NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>36.21±4.45</td>
<td>35.60±4.02</td>
<td>33.43±3.39</td>
<td>37.04±3.42</td>
</tr>
<tr>
<td>Testes (mg)</td>
<td>0.76±0.07</td>
<td>0.72±0.06</td>
<td>0.52±0.08</td>
<td>0.67±0.09</td>
</tr>
<tr>
<td>Seminal vesicles (mg)</td>
<td>0.056±0.03</td>
<td>0.014±0.005</td>
<td>0.040±0.007</td>
<td>0.033±0.008</td>
</tr>
<tr>
<td>Vas deferens (mg)</td>
<td>0.06±0.10</td>
<td>0.06±0.09</td>
<td>0.08±0.09</td>
<td>0.06±0.09</td>
</tr>
</tbody>
</table>
| Values in parentheses are mean±S.D. of eight animals. Values with same superscript in a row do not differ significantly from each other at p>0.01. Values in parentheses are change from control. For calculation of change for NAC and As groups, normal served as control; for As+NAC group, As served as control. NS = not significant. *p<0.05.

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>NAC</th>
<th>As</th>
<th>As+NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (millions/ml)</td>
<td>52.65±4.87</td>
<td>51.94±0.97</td>
<td>34.13±3.93</td>
<td>46.06±5.03</td>
</tr>
<tr>
<td>Viable sperm (%)</td>
<td>70.38±3.54</td>
<td>72.73±3.89</td>
<td>53.25±5.38</td>
<td>63.82±5.41</td>
</tr>
<tr>
<td>Seminal vesicles (%)</td>
<td>60.87±4.67</td>
<td>61.37±5.49</td>
<td>41.69±4.05</td>
<td>54.64±7.04</td>
</tr>
<tr>
<td>HOS tail coiled sperm (%)</td>
<td>61.88±4.67</td>
<td>61.37±5.49</td>
<td>42.75±3.94</td>
<td>56.18±6.80</td>
</tr>
</tbody>
</table>
| Values in parentheses are mean±S.D. of eight animals. Values with same superscript in a row do not differ significantly from each other at p>0.01. Values in parentheses are change from control. For calculation of change for NAC and As groups, normal served as control; for As+NAC group, As served as control. NS = not significant. *p<0.05.
of germ cells and events related to biosynthesis of testosterone. On the other hand, co-treatment of NAC along with As+NAC significantly mitigated the effect of arsenic on the weights of testis and accessory organs. NAC is known for its chelation potential and supplementation of NAC during arsenic intoxication reduced the levels of arsenic in the testis. In consistent with our results, studies of Ramos et al. [45] reported that NAC lowered the levels of arsenic in tissues of rats. It is believed that methyltransferase process is one of the primary mechanisms involved in the detoxification and elimination of arsenic. Methylation involves a cascade of reactions where arsenic is metabolized into monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the presence of S-adenosylmethionine and other methyl donors including glutathione [46,47]. It is well established that NAC is a very effective precursor and stimulator of glutathione synthesis and many disorders, it has been demonstrated that NAC augments glutathione production [48,49].

Studies of Buchet and Lauwerys [50] and Gouris et al. [51] also demonstrated that glutathione stimulates arsenite methylation thereby facilitates its removal in the form of DMA from cells. In the present study, the decreased amount of arsenic in the testes of NAC treated arsenic exposed mice indicates the chelating potential of NAC. Although glutathione is found to be the major contributor in chelating arsenic from tissues, the levels of glutathione in the tissues of arsenic exposed mice still remains to be determined. Testosterone synthesis in Leydig cells and spermatogenesis in seminiferous tubules are the two energy requiring processes in testis. Testosterone secreted from Leydig cells acts on Sertoli cells in seminiferous tubules to create an androgenic stimulation for their normal growth and function [52]. The activity levels of these enzymes were not significantly altered on NAC alone treatment (Table 4).

Discussion
The present study addresses the protective role of NAC on arsenic-induced testicular damage as evidenced by restoration of weights of testis, reduced levels of testicular lipid peroxidation products with increased activities of SOD and catalase, in spermatogenesis and reduced levels of ascorbic acid. In the present study, the animals were exposed to arsenic at a concentration below the observed effect level (NOEL) in order to evaluate if low concentrations of arsenic induce oxidative stress in tissues and suppress the reproduction in male mice. Arsenic was given for 35 days in order to evaluate its effect through a complete spermatogenic cycle, which takes approximately 35 days in mice and the length of spermatogenic cycle is considered as biological constant controlled by germ cell. In the present study, the body weight of the animal exposed to arsenic did not show any significant change, indicating that the general condition of the mice was within normal range. On the contrary, a significant reduction in the weights of testis, epididymis and prostate gland was observed in arsenic exposed mice, which may be due to the inhibition of spermatogenesis and decreased steroidogenesis. It is well known that the weight of testis and accessory sex organs requires continuous androgenic stimulation for their normal growth and function [42].

**Table 3**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>NAC</th>
<th>As</th>
<th>As+NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>38-HSD (nanomoles of NAD+ converted to NADH/mg protein/min)</td>
<td>29.14 ± 1.91</td>
<td>28.79 ± 3.23 (–1.20)</td>
<td>19.15 ± 1.14 (–44.75)</td>
<td>27.72 ± 4.39 (44.75)</td>
</tr>
<tr>
<td>17β-HSD (nanomoles of NADPH converted to NADH/mg protein/min)</td>
<td>24.04 ± 2.26</td>
<td>23.54 ± 3.02 (–2.08)</td>
<td>15.95 ± 2.06 (–33.65)</td>
<td>22.80 ± 3.72 (42.95)</td>
</tr>
<tr>
<td>Testosterone (ng/mL)</td>
<td>9.07 ± 1.04</td>
<td>9.23 ± 0.88 (1.86)</td>
<td>5.12 ± 0.82 (–43.43)</td>
<td>8.13 ± 0.73 (58.79)</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of eight animals. Values with same superscript in a row do not differ significantly from each other at p<0.01. Values in parentheses are % change from control. See calculation of change for NAC and As groups. normal served as control; for As = NAC group. As served as control.

**Table 4**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>NAC</th>
<th>Arsenic</th>
<th>As+NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPX (nanomoles of malondialdehyde formed/g wet wt. of tissue)</td>
<td>14.84 ± 1.92</td>
<td>13.22 ± 1.45 (–11.51)</td>
<td>17.28 ± 1.40 (15.66)</td>
<td>13.83 ± 1.73 (–29.97)</td>
</tr>
<tr>
<td>Catalase (molecules of H2O2 metabolized/mg protein/min)</td>
<td>31.12 ± 5.20</td>
<td>29.89 ± 2.79 (–3.95)</td>
<td>21.14 ± 4.24 (–32.07)</td>
<td>29.22 ± 3.22 (36.32)</td>
</tr>
<tr>
<td>SOD (units/mg protein/min)</td>
<td>1.41 ± 0.10</td>
<td>1.39 ± 0.08 (–1.42)</td>
<td>1.06 ± 0.08 (–24.42)</td>
<td>1.32 ± 0.10 (24.53)</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of eight animals. Values with same superscript in a row do not differ significantly from each other at p<0.01. Values in parentheses are % change from control. See calculation of change for NAC and As groups. normal served as control; for As = NAC group. As served as control.
arsenic exposed mice as compared to control mice. Similar results have also been reported by other investigators [11,52]. The decreased steroidogenic enzyme activity levels may lead to decreased steroidogenesis in experimental mice. Therefore, the decrease in the serum level of testosterone in the mice exposed to arsenic may be due to the decreased steroidogenesis and the decline observed in sperm density as compared to control mice may be the consequence of the reduced testosterone secretion from Leydig cells. Supplementation of NAC has beneficial role on cascade of events associated with steroidogenic enzyme which might be due to protection of Leydig cells against arsenic poisoning.

Sperm plasma membrane, being rich in polyunsaturated fatty acids, is highly susceptible to reactive oxygen species (ROS) attack. To negate the harmful effects of ROS, tests is equipped with a powerful antioxidant defense system involving enzymes like SOD and catalase [53]. SOD is considered the first line of defense against deleterious effects of oxyradicals in the cell by catalyzing the dismutation of superoxide anion radicals to \( \text{H}_2\text{O}_2 \), which is readily degraded by catalase. In the biological system, the antioxidant enzymes catalase protects SOD inactivation by \( \text{H}_2\text{O}_2 \), while the SOD reciprocally protects catalase against inhibition by superoxide anion radicals. Thus, balance of this enzyme system may be essential for preventing lipid peroxidation is well known. NAC has ability to cross cell membrane and the role of NAC in pre-venting lipid peroxidation is well known [54]. The mechanism of ROS induced altered sperm motility is still unclear. However, it is hypothesized that \( \text{H}_2\text{O}_2 \), one of the lipid peroxidation products, might diffuse across the membrane and affect the vital enzymes in the sperms thereby results in decreased sperm motility [55]. It is well recognized that NAC has ability to cross cell membrane and the role of NAC in preventing lipid peroxidation is well known [22]. The present data also demonstrate that co-treatment of NAC significantly mitigated the effects of arsenic-induced oxidative stress in testes of mice by increasing antioxidant enzymes and decreasing lipid peroxidation. In consistent with our results, Modi et al. [25] reported that NAC protects the liver and kidney from arsenic-induced oxidative stress.

It can be concluded from the present results that exposure to arsenic generates ROS by decreasing the activities of antioxidant enzymes and increasing lipid peroxidation thereby causing oxidative stress in testes of mice. This may lead to disruption in the functional integrity of cell organelles. Thus it is suggested that the impairment of spermatogenesis in arsenic exposed mice could me mediated through the induction of oxidative stress in addition to the direct effect on the germinal compartment. The present study also reveals that co-administration of NAC reduces arsenic-induced oxidative stress by decreasing lipid peroxidation and activating antioxidant enzymes in the testes, thereby ameliorating arsenic-induced suppressed reproduction in male mice.

Competing interest

The authors declare that they have no competing interests.

Authors’ contributions

PSR conceived the idea, designed the study, supervised the work, provided the grants for the study and coordinated the work. GPR and SBS carried out the treatment of animals and performed the enzyme assays and sperm analysis. RM and CSs are involved in hormone determinations. PSR and SBS drafted the manuscript for publication. All authors read and approved the final version of manuscript.

Acknowledgements

The authors are grateful to the Head, Department of Biotechnology, S.V. University, Tirupati, India for the encouragement. We thank Prof. K.V.S. Sarma for statistical analysis of data. The research was conducted in accordance with the regulations in the Country and approved by the ethical committee of Sri Venkateswara University, Tirupati, India. We are highly thankful to University Grants Commission, New Delhi (F-3-5/99) and Indian Council of Medical research, New Delhi (5/10/5/2005-RRM) for financial support in the form of research grants to PSR.

References


ACTION REQUIRED: International Journal of Toxicology Contributor Form

GENTERMB@ucmail.uc.edu <GENTERMB@ucmail.uc.edu> Sun, Mar 16, 2014 at 9:32 PM

To: psreddy1955@gmail.com

16-March-2014

Dear Prof. Sreenivasula Reddy,

Your manuscript "Aflatoxin B1-induced reproductive toxicity in male rats: Possible mechanism of action" has been accepted for publication in International Journal of Toxicology.

In order for SAGE to proceed with publication of your article, you must complete a Contributor Form. Under the agreement, you retain copyright to your work and grant an exclusive license to SAGE to publish the article.

You should review and complete the form online at the journal's SAGETRACK site. The following link will take you there directly.

http://mc.manuscriptcentral.com/ijt/URL_MASK=3f2680dc767e40b39074528069a2bba3

Please note that without a completed agreement, we are unable to proceed with publication of your article.

If your library does not currently subscribe to International Journal of Toxicology, please ask them to do so in order to allow your colleagues to access your published article.

If you have any questions please contact the Editorial Office.

With best wishes,

Dr. Mary Beth Genter
GENTERMB@UCMAIL.UC.EDU
International Journal of Toxicology Editorial Office
Dear Prof. Reddy:

Ref: Aflatoxin B1-induced reproductive toxicity in male rats: Possible mechanism of action

I am pleased to accept your paper in its current form for publication in International Journal of Toxicology. The manuscript will now be forwarded to the publisher for copy editing and typesetting.

You will receive proofs in approximately 4-6 weeks. Please watch for a follow-up email which will guide you through our online Copyright Transfer process.

Thank you for your contribution to International Journal of Toxicology, and we look forward to receiving further submissions from you.

Sincerely,
Mary Beth Genter, PhD, DABT, ATS
Editor-in-Chief, International Journal of Toxicology
marybeth.genter@uc.edu
Aflatoxin B1-Induced Reproductive Toxicity in Male Rats: Possible Mechanism of Action

Ch. Supriya¹, B.P. Girish¹,², and P. Sreenivasula Reddy²,*

¹Department of Biotechnology, Sri Venkateswara University, Tirupati – 517502, India

²Department of Zoology, Sri Venkateswara University, Tirupati – 517502, India

Running title: AFB1-induced reproductive toxicity in rats

*to whom correspondence should be addressed at: Department of Zoology
Sri Venkateswara University
Tirupati- 517 502.
Tel: +91-877-2249320
Fax: +91-877-2249611
E-mail: psreddy1955@gmail.com
Abstract

Aflatoxin B1 (AFB1), one of the most common mycotoxins found in human foods is principally hepatotoxic; however, it also affects reproduction. The aim of the present study was to elucidate the reproductive toxic effects and possible mechanism of action of AFB1 in rats. Male Wistar rats were injected intramuscularly with doses of 10, 20, or 50 μg AFB1/Kg body weight on alternate days from 45 to 100 days of age. Significant reductions in body weights, relative weights of reproductive organs, daily sperm production, epididymal sperm count, viable sperm, motile sperm and HOS-tail coiled sperm were observed. Significant decreases in testicular steroidogenic enzymes, serum testosterone levels were also observed indicating decreased steroidogenesis. In silico docking studies illustrated AFB1 binds with steroidogenic acute regulatory protein (StAR) thereby affecting the transport of cholesterol into mitochondria resulting in decreased steroidogenesis.

Keywords: Aflatoxin B1, steroidogenesis, spermatogenesis, serum testosterone, StAR, hydroxysteroid dehydrogenases.
Introduction

In recent years, there is an increasing concern that exposure to toxic pollutants disrupts male reproduction of wild life and humans and plays a pivotal role in the decline of quality and quantity of human semen. The contributing factors for suppressed reproductive health are exposure to environmental contaminants, industrial and occupational chemicals, therapeutics, lifestyle factors and dietary toxins, etc. Aflatoxins are naturally occurring toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* with a characteristic dihydrobisfuran-moiety in their chemical structures. Aflatoxin poisoning can produce recurrent serious health effects which include carcinogenesis, mutagenesis, growth retardation and immune suppression. Aflatoxins are a real threat to the health of humans by their ubiquitous and abiding occurrence in food commodities. AFB1 is metabolized in the intestinal tissue and liver by various microsomal cytochrome P450s, resulting in AFB1-8, 9-epoxide, which binds to DNA forming AFB1-guanine adducts. From earlier studies, it is very clear AFB1 also disrupts the hypothalamo-pituitary-testicular axis resulting in production of defective spermatozoa.

Testosterone biosynthesis occurs in Leydig cells of testes in response to LH synthesized by the pituitary gland. The substrate for steroidogenesis is cholesterol, which is transported into mitochondria by steroidogenic acute regulatory proteins (StAR). In mitochondria, cholesterol is converted into pregnenolone by cytochrome P-450 side chain cleaving enzyme. Later, pregnenolone is transported to smooth endoplasmic reticulum for conversion to testosterone. From earlier studies, it is evident that reduced steroidogenesis is a common feature in aflatoxin mediated reproductive toxicity though the exact mechanism is not known. The present study is intended to elucidate the effects of aflatoxin on steroidogenesis and spermatogenesis by evaluating sperm parameters, circulatory reproductive hormone levels, and activity levels of selected steroidogenic enzymes. Further we have also studied the binding affinity of AFB1 to StAR using *in silico* tools.

Materials and Methods

*Procurement and maintenance of experimental animals*

Male Wistar rats (40 days old; weighing between 90-100 g) were purchased from authorized dealer (Sri Venkateswara Traders, Bengaluru, India). Rats were housed in polypropylene cages (18" x 10" x 8") lined with sterilized paddy husk, and provided filtered tap
water and standard rodent feed (purchased from Sai Durga Agencies, Bengaluru, India) *ad libitum*. The rats were maintained in a well-controlled laboratory conditions (temperature 25 ± 2°C under a schedule of 12-hour light and 12-hour dark cycle, humidity 50 ± 5%). The animals were examined daily for general pathological symptoms. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (Resolution No.03/2011-12/(i)a/CPCSEA/IAEC/SVU/PSR-CHS/Dt. 26-08-2011).

**Chemicals**

Aflatoxin B1 (from *Aspergillus flavus* with 98% purity by HPLC) was purchased from Fermentek Ltd, Jerusalem, Israel. Dehydroepiandrosterone, androstenedione, NADPH and NAD were purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals used in study were of analytical grade and obtained from local commercial sources.

**Experimental design and Necropsy**

Rats were acclimatized to laboratory conditions for one week prior to experiments. Early-pubertal healthy male rats (45 days old) were selected for the present investigation and randomly divided into four groups with 8 animals in each group. The animals in group 1 served as control. Rats in groups 2, 3 and 4 were injected with AFB1 at a dose of 10, 20 and 50μg/Kg body weight, respectively, dissolved in DMSO, on alternative days through intra-muscular route for 60 days. The dosage levels of Aflatoxin B1 were based on previous studies on rats, which caused reproductive abnormalities.18,19,24

After completion of the experimental period the rats were fasted overnight, weighed, and killed by cervical dislocation. Testes, epididymis (caput, corpus, cauda), prostate gland, vas deferens, and seminal vesicles were quickly removed, cleared of the adhering tissues and weighed wet to the nearest milligram and used for the determination of tissue somatic indices (TSI).

\[ \text{TSI} = \left( \frac{\text{weight of the tissue (g)}}{\text{body weight of the animal (g)}} \right) \times 100 \]

Testes were used for determination of daily sperm production, biochemical analysis, and enzyme assays and epididymis was used for sperm analysis.

**Analysis of epididymal sperm**

Epididymal sperm were obtained by chopping cauda epididymis in physiological saline (0.9% NaCl in distilled water). The sperm density was determined by using a Neubauer Chamber,
as described by Belsey et al.25. The data were expressed as millions/ml. Progressive sperm motility was evaluated by the method described earlier25, within 5 min following their isolation from cauda epididymis at 37°C and the data were expressed as percent motility. The viability of the sperm was determined using 1% trypan blue reagent26. The sperm membrane integrity was determined by exposing the sperm to hypoosmotic solution and observed for sperm with coiled tail under the microscope (Hypoosmotic swelling (HOS) tail coiling) and the percent of sperm with coiled tail was estimated using the method described earlier.27

Daily Sperm Production

Daily sperm production was determined in the testis by the method of Blazak et al.28. Briefly, the testes were decapsulated and homogenized in 50 ml of ice cold 0.9% NaCl solution containing 0.01% Triton X-100 using a sterilized mortar and pestle. After thorough mixing of homogenate, the number of sperm heads was counted in Neubauer haemocytometer. The number of sperm produced per gram testicular tissue per day was calculated.

Assay of testicular steroidogenic marker enzymes

The testicular tissue was homogenized in ice-cold Tris–HCl buffer (pH 6.8). The microsomal fraction was separated and used as enzyme source. The activity levels of 3β hydroxysteroid dehydrogenase (3β HSD) (EC 1.1.1.51) and 17β hydroxysteroid dehydrogenase (17β HSD) (EC 1.1.1.64) were determined by the method of Bergmayer29. The enzyme assays were made under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration. Protein content in the enzyme source was estimated by the method of Lowry et al.30 using bovine serum albumin as standard. The enzyme activities were expressed as nmol of NAD converted to NADH/mg protein/min (3β HSD) or nmol of NADPH converted to NADP/mg protein/min (17β HSD).

Determination of Serum Hormone levels

A blood was collected from the heart of each animal before necropsy using a heparinized syringe. The serum was separated by centrifugation at 2,000 x g for 15 min after overnight storage at 4°C and stored at -20°C until all of the samples were collected. Radio Immuno Assay (RIA) for serum testosterone was performed by the method of Rao et al.31. The sensitivity of the assay was calculated as 0.002 ng and intraassay variation was found to be 6.5%. Serum FSH and LH levels were determined according to the method of Lin et al.32. Iodination of rFSH and rLH with 125I was performed by the method of Greenwood et al.33 using Chloramine-T as an oxidizing
agent. The sensitivity of the assay was calculated as 0.008 ng for FSH and 0.006 ng for LH. The intraassay variations were 5.8% and 6.9% for FSH and LH, respectively. All of the samples were run at the same time to avoid interassay variation.

Docking Studies

Molecular docking studies were performed with AFB1 and cholesterol against StAR protein (PDB ID: 3POL) using PyRx virtual screening software, which uses AutoDock Vina for docking studies. The interaction between protein and ligands was interpreted and visualized using PyMOL.

Statistical analysis

The data were statistically analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey’s test using SPSS 16.0. The data were expressed as mean ± S.D. and ‘p’ value <0.05 was considered significant. All statistical tests were performed using Statistical Package for Social Sciences 16.0 (SPSS Inc., Chertsey, UK).

Results

Body weights and tissue indices

Body weights of the control and AFB1 treated rats are presented in Table 1. Significant decreases in body weights were found in AFB1 treated rats when compared to controls. Significant decreases in the indices of testis and accessory sex organs were also observed at all dosages with the exception of the testes, corpus epididymis and prostate at 10 μg/kg compared with controls (Table 1). Food consumption (g/kg body weight/day) was almost similar in all groups (authors’ unpublished data).

Sperm parameters

The effect of AFB1 on testicular daily sperm production (DSP), cauda epididymal sperm count, sperm motility, sperm viability, and sperm HOS coiling in rats are given in Table 2. A significant decrease in DSP, sperm count, sperm motility, sperm viability and sperm coiling percentage was observed in rats exposed to selected dosages of AFB1 when compared to controls with the exception of DSP at 10 μg.

Testicular steroidogenic marker enzymes and Serum hormone profiles

Table 3 summarizes the data on the activity levels of 3β HSD and 17β HSD in the testis of control and AFB1 exposed rats. The activity levels of testicular 3β HSD and 17β HSDs were
significantly decreased in rats exposed to AFB1 in a dose-dependent manner when compared to control rats (Table 3). Furthermore, a dose-dependent reduction in serum testosterone levels was also observed in rats exposed to AFB1 (Table 4). However, the levels of FSH and LH increased significantly in serum of rats treated with AFB1 when compared with the control rats (Table 4).

Docking studies

AFB1 and cholesterol binding affinities with StAR (PDB ID: 3POL) were evaluated using docking studies. AFB1 and cholesterol interacts with GLN 262, LEU245, PHE184, GLU169, LEU247, ARG182, PRO181, ALA172, GLU173, LEU178, LEU260, THR263, VAL259 and ALA172 at the binding site of StAR. Besides these interactions, hydrogen bonds with GLN262 with bond lengths of 2.8 and 2.0 were observed with AFB1 (Figure 1). Aflatoxin B1 was found to possess high binding affinity (-8.1 kcal/mol) when compared with natural ligand, cholesterol (-7.8 kcal/mol).

Discussion

In the present study, male rats were exposed to sub-lethal dosages (10, 20, or 50μg/kg body weight) of AFB1 from early puberty (40 days of age) to sexual maturity (100 days of age), in order to evaluate its effect through a complete spermatogenic cycle, which takes approximately 60 days in rats. The exposure of rats to 10, 20 or 50 μg/kg body weight corresponds to human exposure in areas with high AFB1 contamination leading to outbreak of aflatoxicosis. In two instances of aflatoxicosis, the daily AFB1 intake was estimated to have been at least 38 μg and 55 μg/kg body weight for an undetermined number of days.

The present work has shown that exposure of rats to 10, 20, or 50μg/kg body weight of AFB1 significantly decreased the body weight gain and the relative weights of testis and other reproductive organs. The decrease in the weight of the testes may be due to degeneration of germinal epithelium. The observed loss in weight of epididymis may be due to decrease in sperm production. Similar effects were observed in male rats exposed to AFB1 by other researchers which may be due to reduced bioavailability and/or production of androgen in the AFB1 exposed rats. It is well known that the morphology and functional integrity of testes and accessory sex tissues are dependent on the bio-availability of androgens. The weight of reproductive organs has also been used as a sensitive bioassay of androgens. Decrease in the serum level of testosterone may be a reason behind the significant decline observed in the weight
of testis and accessory sex tissues in rats exposed to AFB1. The data also reveal reduced levels of circulatory testosterone in rats exposed to AFB1, indicating a possible inhibition of androgen synthesis in experimental rats.

The effects of AFB1 on spermatogenesis were assessed by determining daily sperm production and epididymal sperm quality and density. The epididymal sperm count and daily sperm production were also used as important indicators to detect the reproductive toxic effects of various chemicals. It is well known that production and maturation are important for the development of healthy spermatozoa. The composition of the internal epididymal milieu is responsible for sperm maturation, which is under the control of androgen. In rats, androgen-binding protein (ABP) secreted by Sertoli cells into the lumen of seminiferous tubules, under FSH stimulation, is transported to the epididymis, where it accumulates at concentrations higher than those found in the testes. This leads to a high local concentration of androgens, essential for maturation of epididymal spermatozoa. The effects seen in this study such as decreased quantity and deteriorated quality of sperm have been reported in rats and mice chronically treated with sub-lethal doses of AFB1. It is well known that testosterone plays a pivotal role in the regulation of structural and functional integrity of reproductive organs.

To determine whether AFB1 affects testosterone biosynthesis, we measured serum testosterone and determined the activity levels of marker steroidogenic enzymes in testes. Steroidogenesis is a complex testicular process where cholesterol is converted to testosterone by a cascade of enzymes. In response to LH secreted by the pituitary gland, transcription of genes involved in testosterone biosynthesis in Leydig cell is induced. Among them, StAR is an important rate limiting protein involved in the transport of cholesterol from cytosol to mitochondria. In mitochondria, cholesterol is converted into pregnenolone and then transported to smooth endoplasmic reticulum for further steps involved in testosterone biosynthesis.

Verma and Nair observed a significant increase in cholesterol concentrations in testes of AFB1 treated mice which may be due to incomplete utilization of cholesterol or impaired steroidogenesis. Similar decrease in serum testosterone level has been reported in rats following exposure to AFB1. FSH and LH act on Sertoli and Leydig cells and thus regulate spermatogenesis and steroidogenesis, respectively. The decrease in serum testosterone levels could be due to diminished responsiveness of Leydig cells to LH and/or direct inhibition of testosterone synthesis in rats exposed to AFB1. The increase in serum FSH levels indicates an
impairment of spermatogenesis in AFB1 treated rats and reflects germ cell loss or damage to Sertoli cells, thereby affecting the feedback regulation of FSH secretion.\textsuperscript{49} Hasanzadeh \textit{et al.}\textsuperscript{50} observed a significant increase in serum levels of FSH with a decrease in testosterone in AFB1 treated rats when compared to controls. The lowered levels of circulatory testosterone with elevated levels of FSH and LH indicate an intact pituitary-testicular axis in AFB1 treated rats.

To determine the mechanism of action of AFB1, we performed docking studies between StAR and AFB1. Earlier studies have shown that exposure to AFB1 induces swelling of mitochondria and dysfunction due to accumulation of calcium during aflatoxicosis has also been reported.\textsuperscript{51-52} Moreover, aflatoxin metabolism occurs in mitochondrion resulting in mitochondrial swelling which suggests AFB1 interaction with StAR protein. It is evident from the present study, AFB1 possess high binding affinity when compared with natural ligand, cholesterol. Similar binding sites and affinities were observed with other StAR domain containing proteins \textit{[Mus musculus] (1JSS)} with binding affinity -9.0 kcal/mol and \textit{Homo sapiens} (2PSO) with binding affinity -8.6 kcal/mol and -8.9 kcal/mol for AFB1 and cholesterol respectively. From the data, it is evident that AFB1 competes with cholesterol thereby affects the cholesterol transport into the mitochondria by StAR. The reduced cholesterol levels in the mitochondria might be responsible for the decreased steroidogenesis and spermatogenesis.

In conclusion, the results of the study suggest that AFB1 binds competitively to StAR thereby affecting cholesterol transport into mitochondria resulting in reduced biosynthesis of testosterone. Since testosterone is very much essential for spermatogenesis, the decreased circulatory testosterone levels affect daily sperm production and quality of the sperm. Whether the decrease in sperm count and quality affect fecundity of AFB1 treated rats cannot be determined from the present data. Hence, additional in-depth fertility studies are needed involving artificial insemination using a fixed number of sperm from the cauda epididymis and also using the male’s ability to sire offspring in a fixed-time period. These studies will help us to determine the effect of AFB1 not only on the status of male libido but also on the fecundity of male rat. Our findings are significant because humans and animals are continuously exposed to aflatoxins and thus may be more prone to higher incidence of reproductive disorders. Finally, the data presented in this study provide strong evidence that AFB1 is a potent endocrine disruptor \textit{in vivo}.

\textbf{Ethical standards}
Animal handling and experiments were in accordance with the guidelines prepared by the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (Resolution No.03/2011-12/i’a/CPCSEA/IAEC/SVU/PSR-CHS/Dt. 26-08-2011).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The present study was supported financially by the University Grants Commission through MRP grant to PSR. CHS and BPG were supported by a UGC-RGNF and CSIR-JRF respectively.

Author contributions

PSR conceived the idea, participated in its design, supervised the work, evaluated the data and coordinated the study. CHS and BPG treated the animals and maintained the rat colony. CHS carried out sperm analyses and enzyme assays. BPG performed in silico studies. PSR determined the hormone levels. PSR, CHS and BPG drafted the manuscript for publication. All authors read and approved the final manuscript.

References


28. Blazak WF, Treinen KA, Juniewicz PE. Application of testicular sperm head counts in


FIGURE LEGEND

Figure 1. Mesh Model of StAR Showing Hydrophobic Binding Pocket of Cholesterol (A) and AFB1 (B).
### Table 1. Effect of Aflatoxin B1 on Body Weights (g) and Tissue Indices (g %) in Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>10 µg</th>
<th>20 µg</th>
<th>50 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>272±16.15</td>
<td>249.5±7.74</td>
<td>216±6.89</td>
<td>193.8±12.7</td>
</tr>
<tr>
<td></td>
<td>(-6.62)</td>
<td>(-20.59)</td>
<td>(-28.75)</td>
<td></td>
</tr>
<tr>
<td>Testes</td>
<td>1.161±0.025</td>
<td>1.157±0.07</td>
<td>1.058±0.04</td>
<td>0.99±0.06</td>
</tr>
<tr>
<td></td>
<td>(-0.34)</td>
<td>(-8.87)</td>
<td>(-14.73)</td>
<td></td>
</tr>
<tr>
<td>Cauda epididymes</td>
<td>0.226±0.012</td>
<td>0.204±0.022</td>
<td>0.17±0.021</td>
<td>0.094±0.007</td>
</tr>
<tr>
<td></td>
<td>(-9.73)</td>
<td>(-24.78)</td>
<td>(-58.41)</td>
<td></td>
</tr>
<tr>
<td>Caput epididymes</td>
<td>0.962±0.045</td>
<td>0.935±0.033</td>
<td>0.916±0.021</td>
<td>0.741±0.065</td>
</tr>
<tr>
<td></td>
<td>(-2.81)</td>
<td>(-4.78)</td>
<td>(-22.97)</td>
<td></td>
</tr>
<tr>
<td>Corpus epididymes</td>
<td>0.026±0.003</td>
<td>0.023±0.001</td>
<td>0.023±0.003</td>
<td>0.022±0.002</td>
</tr>
<tr>
<td></td>
<td>(-11.54)</td>
<td>(-11.54)</td>
<td>(-15.38)</td>
<td></td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>0.467±0.042</td>
<td>0.422±0.038</td>
<td>0.407±0.025</td>
<td>0.224±0.004</td>
</tr>
<tr>
<td></td>
<td>(-9.63)</td>
<td>(-12.85)</td>
<td>(-52.03)</td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>0.145±0.007</td>
<td>0.133±0.016</td>
<td>0.125±0.012</td>
<td>0.097±0.013</td>
</tr>
<tr>
<td></td>
<td>(-8.27)</td>
<td>(-13.79)</td>
<td>(-33.10)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 8 individuals.

Values in the parentheses are percent change from that of control.

*Values are significantly different from control at p<0.05.
Table 2. Effect of Aflatoxin B1 on Testicular Daily Sperm Production and Epididymal Sperm Parameters in Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>10 µg</th>
<th>20 µg</th>
<th>50 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily Sperm Production (millions/g testis)</td>
<td>21.02±4.86</td>
<td>18.20±2.31</td>
<td>10.59±1.93</td>
<td>7.76±1.85</td>
</tr>
<tr>
<td></td>
<td>(-13.41)</td>
<td>(-49.62)</td>
<td>(-63.08)</td>
<td></td>
</tr>
<tr>
<td>Sperm Count (millions/ml)</td>
<td>65.87±5.09</td>
<td>41.72±4.49</td>
<td>30.07±5.24</td>
<td>21.52±3.0</td>
</tr>
<tr>
<td></td>
<td>(-36.66)</td>
<td>(-54.35)</td>
<td>(-67.33)</td>
<td></td>
</tr>
<tr>
<td>Motility (%)</td>
<td>76.0±4.5</td>
<td>50.63±3.79</td>
<td>39.07±5.95</td>
<td>28.07±4.0</td>
</tr>
<tr>
<td></td>
<td>(-33.42)</td>
<td>(-48.68)</td>
<td>(-63.16)</td>
<td></td>
</tr>
<tr>
<td>Viability (%)</td>
<td>77.0±4.58</td>
<td>60.37±5.35</td>
<td>48.07±4.16</td>
<td>38.07±3.10</td>
</tr>
<tr>
<td></td>
<td>(-21.69)</td>
<td>(-37.66)</td>
<td>(-50.65)</td>
<td></td>
</tr>
<tr>
<td>HOS-tail coiled sperm (%)</td>
<td>60.23±6.59</td>
<td>29.17±3.59</td>
<td>15.07±1.87</td>
<td>13.27±3.11</td>
</tr>
<tr>
<td></td>
<td>(-51.68)</td>
<td>(-75.09)</td>
<td>(-78.08)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 8 individuals
Values in the parentheses are percent change from that of control.
*Values are significantly different from control at p<0.05.
Table 3. Effect of Aflatoxin B1 on Testicular 3β- and 17β Hydroxysteroid Dehydrogenase Activity Levels in the Testes of Male Rats

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>10µg</th>
<th>20µg</th>
<th>50µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>3β-HSD</td>
<td>32.0±4.15</td>
<td>21.7±3.12</td>
<td>9.7±2.12</td>
<td>6.3±1.2</td>
</tr>
<tr>
<td>(n moles of NAD converted to NADH/mg. protein/min)</td>
<td>(-32.19)</td>
<td>(-69.69)</td>
<td>(-80.31)</td>
<td></td>
</tr>
<tr>
<td>17β-HSD</td>
<td>21.9±3.91</td>
<td>17.3±2.2</td>
<td>4.3±0.74</td>
<td>2.8±0.513</td>
</tr>
<tr>
<td>(n moles of NADPH converted to NADP/mg. protein/min)</td>
<td>(-21.0)</td>
<td>(-80.36)</td>
<td>(-87.21)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 8 individuals.
Values in the parentheses are percent change from that of control.
*Values are significantly different from control at p<0.05.
**Table 4.** Effect of Aflatoxin B1 on Serum Testosterone, FSH and LH Levels in Rats

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control</th>
<th>10 µg</th>
<th>20 µg</th>
<th>50 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (ng/mL)</td>
<td>7.96±1.21</td>
<td>5.44*±1.02</td>
<td>5.09*±0.91</td>
<td>3.66*±0.46</td>
</tr>
<tr>
<td></td>
<td>(-31.66)</td>
<td>(-36.05)</td>
<td>(-54.02)</td>
<td></td>
</tr>
<tr>
<td>FSH (ng/mL)</td>
<td>5.66±0.71</td>
<td>9.23*±1.18</td>
<td>13.73*±2.69</td>
<td>19.71*±3.14</td>
</tr>
<tr>
<td></td>
<td>(63.07)</td>
<td>(142.58)</td>
<td>(248.23)</td>
<td></td>
</tr>
<tr>
<td>LH (ng/mL)</td>
<td>1.68±0.09</td>
<td>2.96*±0.14</td>
<td>6.88*±0.45</td>
<td>8.82*±0.79</td>
</tr>
<tr>
<td></td>
<td>(76.19)</td>
<td>(309.52)</td>
<td>(425.0)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 8 individuals.  
Values in the parentheses are percent change from that of control.  
*Values are significantly different from control at p<0.05.