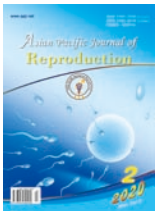

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Effect of aqueous seed extract of *Mucuna pruriens* on arsenic-induced testicular toxicity in mice

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ABSTRACT

Objective: To investigate the protective effects of *Mucuna (M.) pruriens* against arsenic-induced testicular impairment in albino mice.

Methods: Thirty-six male albino mice were divided into six groups, with 6 mice in each group. Group 1 received drinking water as the normal control. Groups 2 to 6 received sodium arsenite (40 mg/L) in drinking water. Group 3 received sodium arsenite (40 mg/L) and 300 mg/kg body weight (b.w.) of *N*-acetylcysteine. Group 4 received sodium arsenite (40 mg/L) and 500 mg/kg b.w. of *M. pruriens*. Group 5 received sodium arsenite (40 mg/L) and 1 000 mg/kg b.w. of *M. pruriens*. Group 6 received sodium arsenite (40 mg/L) and 2 000 mg/kg b.w. of *M. pruriens*. *N*-acetylcysteine and *M. pruriens* were administered orally once a day. Animals were subjected to the above treatments for 45 days. Animals were sacrificed with overdose of ketamine 24 h following drug administration. The testis was used for biochemical estimations (lipid peroxidation and glutathione), and the epididymis was used to determine the sperm count and morphology.

Results: Sodium arsenite significantly decreased (P<0.01) the sperm count and glutathione levels of the testis. It significantly increased (P<0.01) the abnormal architecture of the spermatozoa and lipid peroxidation levels. Treatment with *M. pruriens* significantly increased the sperm count and the glutathione levels [500 mg/kg b.w. and 1 000 mg/kg b.w. (P<0.01)]. A significant decrease in sperm abnormality and lipid peroxidation levels [500 mg/kg b.w. and 1 000 mg/kg b.w. (P<0.01)] was also observed.

Conclusions: The perturbed sperm parameters and antioxidant levels of the arsenic insulted testis are attenuated by 500 mg/kg b.w. and 1 000 mg/kg b.w. *M. pruriens.*

KEYWORDS: Sodium arsenite; *Mucuna pruriens*; Sperm morphology; Sperm count; Testis

1. Introduction

Panoptical use of metals advanced human civilization growth. Metals are naturally occurring and ubiquitous within the environment. Moreover, the evolution of life occurred in the presence of metals, and many metals are essential for normal biological processes. Although some metals are toxic with increasing exposure, organisms have to deal with the toxicity of these omnipresent metals. Further, toxic metals are increased in the environment due to urbanization and industrialization. It has been reported that environmental toxicants may be associated with male infertility by the decrease in the quality of semen during the last decade[1,2].

Arsenic, a proven genotoxic carcinogen is an omnipresent metalloid. It is released in the environment from various sources. It is used in semiconductors, glassware, alloys, and wood preservatives. Many reports reveal the presence of high content of inorganic arsenic in groundwater from different parts of the world, including India and Bangladesh[3,4].

Epidemiologic studies have documented that exposure to arsenic is linked with male reproductive toxicity[5,6]. This study was supported by Ommati *et al*[7] who could connect the link between arsenic and male infertility. Reproductive health is a condition of complete mental, physical, and social wellness, as referred by World Health Organization. It becomes a vital feature of

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general health and human development. If it is not maintained at present, it can affect the health of the next generation.

Drinking water consists of arsenic in mainly inorganic form, and it is more harmful than organic arsenic forms. Inorganic arsenic exists as a trivalent form, arsenite (As^{3+}), which is more active and toxic than pentavalent form, arsenate (As^{5+}). The permissible limit for arsenic in drinking water is 10 ppb (World Health Organization). Vomiting, abdominal pain, and severe diarrhea are a few symptoms of acute arsenic poisoning[8]. Chronic ingestion of arsenic results in its accumulation in vital organs, and tissues, leading to atherosclerosis, hypertension[9,10]. Studies have shown that arsenic intoxication results in testicular regression in mice[11,12]. Testicular degeneration and tissue necrosis have been reported[13,14]. A decrease in sperm count and motility has been demonstrated in a dose-dependent manner after arsenic treatment[11,15].

Chelation therapy in arsenic toxicity helps in the elimination of arsenic from the body, thus preventing the hazardous effects of arsenic. Agents used for arsenic chelation are dimercaptosuccinic acid and *D*-penicillamine. The clinical outcome of these drugs and long term use of such medicines in arsenic chelation is mystifying.

N-acetylcysteine, a precursor to glutathione acts as a biological antioxidant. It is used in the treatment of arsenic poisoning[16]. Tissue injury in various organs is prevented due to its ability to scavenge oxygen free radicals. The antioxidant property is due to the presence of a sulfhydryl group.

Mucuna (M.) pruriens is a leguminous plant. It has an anti-lipid peroxidation property[17]. *M. pruriens* seeds contain alkaloids and saponins, which increase the testicular weight and development of sperms[18]. *M. pruriens* acts by reducing the levels of free radicals, thus increasing the germ cell number eventually[19]. *M. pruriens* is recognized to increase testosterone levels[20], thus improving semen quality in infertile men[21]. The advantage of plant extracts is that they are less expensive to produce and are affordable to the poor population who need them the most. They can be grown locally.

A few studies supported the pro-male fertility properties of *M. pruriens*[22,23]. Although the exact mechanism is obscure, it is presumed that this activity may be due to the other properties of *M. pruriens*, *i.e*., antioxidant potential, adaptogenic, and nutritional property of *M. pruriens*. The protective role of *M. pruriens* in arsenic-induced gonadotoxicity is not much studied. Based on the promising pro-male fertility property and other nutritional properties of *M. pruriens*, we attempted the present study to evaluate its potential in protecting the spermatogenic loss, and its role in the antioxidant level of the testis.

2. Materials and methods

2.1. Chemicals

Sodium arsenite anhydrous (98.5%) was obtained from Nice chemicals (P) Ltd. *N*-acetylcysteine (Samarth life sciences private limited) was obtained from a medical store at Udupi. Thiobarbituric acid, trichloroacetic acid and 5,5'-dithiobis-2-nitrobenzoic acid were obtained from Durga laboratories, Mangalore, India.

2.2. Plant extract preparation

The identification of *M. pruriens* was carried out by the Faculty of the Pharmacognosy Department (Specimen No: SDM/954/17112301). Seeds of *M. pruriens* were collected from Udupi and cleaned. Seeds of *M. pruriens* were ground into a fine powder. 50.0 g of the powder was added to 100 mL distilled water at 4 ℃ for 24 h. Centrifugation of the suspension was carried out at 10 000 *g* for 25 min. The supernatant was lyophilized to powder and was stored at -4 ℃. It was prepared freshly for use[24]. The yield from the extract was 15.8 g.

2.3. Animals and housing

Thirty-six male albino mice [9-12 weeks old, weighing (28±2) g] that were opted for the study were bred locally in the animal house at Manipal Academy of Higher Education. They were housed in cages with appropriate bedding, standard temperature (22–24) ℃, light-dark cycle (12 h/12 h), and relative air humidity (40%–60%). The animals were acclimated to the conditions in the laboratory for seven days before the commencement of the experiment. Animals were fed with rat pellets and water.

2.4. Experimental design

The 36 male albino mice were divided into six groups, with 6 mice in each group. Group 1 received drinking water as the normal control. Groups 2 to 6 received sodium arsenite (40 mg/L) in drinking water[17]. Group 3 received sodium arsenite (40 mg/L) and 300 mg/kg body weight (b.w.) of *N*–acetylcysteine[25]. Group 4 received sodium arsenite (40 mg/L) and 500 mg/kg b.w. of *M. pruriens*. Group 5 received sodium arsenite (40 mg/L) and 1 000 mg/kg b.w. of *M. pruriens*. Group 6 received sodium arsenite (40 mg/L) and 2 000 mg/kg b.w. of *M. pruriens*[26]. *N*-acetylcysteine and *M. pruriens* were administered orally once/day. Animals were subjected to the above treatments for 45 days[11]. Animals were sacrificed with overdose of ketamine 24 h following drug administration. Left testis was used for biochemical estimations, and epididymis was used to determine the sperm count and morphology.

2.5. Epididymal sperm count

Freshly prepared phosphate-buffered saline (1 mL, pH 7.2) was used for mincing the epididymis. A little amount of the suspension was diluted in phosphate-buffered saline in the ratio of 1:40. Neubauer's chamber was then charged with the sample of the diluted suspension. Eight large squares (apart from the erythrocyte area) were used for counting the sperm and multiplied by 50 000 to get the total count.

2.6. Sperm morphology assay

Freshly prepared phosphate-buffered saline (1 mL, pH 7.2) was used for mincing the epididymis. A part of this suspension was mixed with 0.2 mL of 1% aqueous eosin. It was kept aside for 30 min following which a drop of the suspension was placed at one end of the slide. Smears were prepared. The slides were air-dried and viewed under a microscope (40×, Magnus MLX) for abnormality in the shape of the sperms. One thousand sperms per animal were scored. Head and tail abnormalities were noted.

2.7. Preparation of tissue homogenate

Testicular tissue (1 g) was minced. It was added to the manual homogenizer containing 10 mL of 10 mmol cold potassium phosphate buffer (pH 7.4). The suspension containing the minced tissue was centrifuged at 10 000×*g* for 10 min to remove the cell debris and the intact cells. The supernatant solution was used for estimating the levels of lipid peroxidation and the content of glutathione.

2.7.1. Determination of lipid peroxidation level

To 20 μL testis homogenate sample, 200 μL 0.67% thiobarbituric acid and 100 μL 20% trichloroacetic acid were added and incubated at 100 ℃ for 20 min. Then, it was centrifuged at 12 000 rpm for 5 min and 100 μL of supernatant was transferred to a 96-well micro test plate. Optical density of supernatant was read at 540 nm by using an ELISA reader (Bio Tek Instruments ELX800-MS, USA).

2.7.2. Determination of reduced glutathione level

Mixture of 100 μL of testis tissue homogenate and 5% trichloro acetic acid (100 μL) solution was centrifuged at 5 000 rpm for 5 min. 25 μL of tissue supernatant, sodium phosphate buffer (PBS 0.2 M, pH 8.0) (150 μL) and 5,5'-dithiobis-2-nitrobenzoic acid (0.6 mM) (25 μL) was added together into a micro test plate containing 96 wells. It was incubated for 10 min at room temperature. Absorbance was read at 412 nm by using an ELISA reader (Bio Tek Instruments ELX800-MS, USA).

2.8. Data analysis

The uniform data that were generated were expressed as mean±standard deviation (mean±SD). One-way analysis of variance followed by Tukey's *post hoc* test was used for the analysis. Version 5 of Graph pad prism was used. A *P*-value of less than 0.05 was considered as statistically significant.

2.9. Ethical approval

This study was approved by the Institutional Animal Ethics Committee (ethical approval number: IAEC/KMC/52/2015). Maintenance of animals was done according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India for the use of laboratory animals.

3. Results

3.1. Effect of M. pruriens on sperm indices

There was a significant reduction in sperm count ($P<0.01$) after treatment with sodium arsenite in comparison to the normal control group. Co-administration of *M. pruriens* (500 mg/kg b.w., 1 000 mg/kg b.w.) and sodium arsenite significantly increased the sperm count ($P<0.01$) in comparison to the sodium arsenite treated group. The group treated with *M. pruriens* 2 000 mg/kg b.w. did not show any increase in sperm count when compared to sodium arsenite treated group. Groups treated with *M. pruriens* (500 mg/kg b.w., 2 000 mg/kg b.w.) along with sodium arsenite showed a significant decrease in sperm count when compared to the *M. pruriens* + *N*-acetylcysteine group. Animals treated with *M. pruriens* 1 000 mg/kg b.w. along with sodium arsenite showed increased sperm count in comparison to *M. pruriens* + *N*-acetylcysteine group, but the result was not statistically significant (Table 1)

The spermatozoa of the group treated with sodium arsenite showed significant higher percentages of sperm abnormality ($P<0.01$) in comparison to the normal control group. Co-administration of both doses of *M. pruriens* (500 mg/kg b.w., 1 000 mg/kg b.w.) and sodium arsenite significantly decreased the number of abnormal sperms ($P<0.01$) in comparison to the group treated with sodium arsenite alone. The group treated with *M. pruriens* 2 000 mg/kg b.w. did not show any change in comparison to the sodium arsenite treated group. The number of abnormal sperms was significantly higher in groups co-administered with *M. pruriens* (500 mg/kg b.w., 2 000 mg/kg b.w.) and sodium arsenite when compared to the group treated with *M. pruriens* + *N*-acetylcysteine. There was a significant reduction in the number of abnormal sperms in the group treated with 1 000 mg/kg b.w. of *M. pruriens* along with sodium arsenite when compared to the *M. pruriens* + *N*-acetylcysteine treated group.

3.2. Effect of M. pruriens on lipid peroxidation levels

Lipid peroxidation levels were higher in the group treated with sodium arsenite in comparison to the normal control group. Co-administration of *M. pruriens* [500 mg/kg b.w. and 1 000 mg/kg b.w. ($P<0.01$)] and sodium arsenite significantly reduced the levels of lipid peroxidation in the sodium arsenite alone treated group. The group treated with *M. pruriens* 2 000 mg/kg b.w. did not show any change in comparison to the sodium arsenite treated group. Groups treated with *M. pruriens* (500 mg/kg b.w. and 2 000 mg/kg b.w.) along with sodium arsenite showed marginally higher lipid peroxidation

Table 1. Effect of *M. pruriens* on the sperm count, sperm morphology, lipid peroxidation and glutathione levels of arsenic treated mice.

Parameters	Normal control	SA control	SA+NAC	SA+MP (500)	SA+MP (1 000)	SA+MP (2 000)
Sperm count (millions/mL)	14.65±0.45	5.83±0.40*	13.35±0.69#	9.83±1.44#△	13.46±1.52#	6.49±0.31△
Sperm abnormality (%)	9.90±1.14	41.48±2.56*	21.23±0.93#	26.73±1.27#△	16.20±2.43#△	39.92±2.41△
TBARS levels (nanomoles/mL of tissue homogenate)	70.69±2.45	100.9±4.58*	82.25±10.3#	85.00±9.51#	76.89±5.82#	92.85±4.87
Glutathione levels (nanomoles/mL of tissue homogenate)	0.95±0.11	0.62±0.04*	0.88±0.03#	0.80±0.05#	0.90±0.12#	0.64±0.06△

Values are expressed as mean±SD; n=6 in each group. *P<0.01: compared to the normal control group; #P<0.01: compared to the SA control group; △P<0.01: compared to the SA+NAC group. SA: sodium arsenite; NAC: *N*-acetylcysteine; MP (500): *M. pruriens* aqueous seed extract 500 mg/kg body weight; MP (1 000): *M. pruriens* aqueous seed extract 1 000 mg/kg body weight; MP (2 000): *M. pruriens* aqueous seed extract 2 000 mg/kg body weight; TBARS: thiobarbituric acid reactive substances.

levels in comparison to *M. pruriens* + *N*-acetylcysteine treated group. Animals treated with 1 000 mg/kg b.w. of *M. pruriens* along with sodium arsenite showed a slight decrease in lipid peroxidation levels when compared to *M. pruriens* + *N*-acetylcysteine treated animals (Table 1).

3.3. Effect of M. pruriens on glutathione levels

The sodium arsenite treated group showed a significant decrease in levels of glutathione when compared to the normal control group. Co-administration of sodium arsenite and *M. pruriens* (500 mg/kg b.w. and 1 000 mg/kg b.w.) showed a significant rise in glutathione levels. The group treated with *M. pruriens* 2 000 mg/kg/b.w. did not show any change with respect to the sodium arsenite treated group. A significant reduction in the level of glutathione was observed in the group treated with *M. pruriens* 2 000 mg/kg b.w along with sodium arsenite (P<0.01) when compared to the group treated with *M. pruriens* + *N*-acetylcysteine. Sodium arsenite + *M. pruriens* 1 000 mg/kg b.w. showed a slight increase in the levels of glutathione in comparison to *M. pruriens* + *N*–acetylcysteine treated group (Table 1).

4. Discussion

The data generated indicate that arsenic exposure significantly decreased the sperm number and increased the number of abnormal sperms. Earlier studies have shown that exposure to arsenic caused male reproductive toxicity when administered by means of drinking water[27,28]. Treatment with sodium arsenite showed a significant decrease in relative testicular weight, accessory sex organ weights, and epididymal sperm count. The seminiferous tubules' diameter and the sperm quality were suppressed by arsenic[29]. Mukyopadyay *et al* had observed a decrease in serum testosterone levels along with significant diminution in testicular glutathione *S*-transferase activity and reduced glutathione level[30]. Arsenic exposure causes increased production of free radicals, thus affecting the sperm production in the testes and its retention in seminiferous tubules. Arsenic also reduces the levels of follicle-stimulating hormone and luteinizing hormone, leading to decreased testosterone production[31,32]. Compounds containing arsenic were reported to cause DNA damage by reactions involving free radicals resulting in defective sperms[28]. In this study, arsenic exposure reduced the glutathione levels, and concomitantly increased the lipid peroxidation levels in the testis.

Similarly, arsenic has been reported to reduce the levels of glutathione in the testis and increase the levels of lipid peroxidation[33,34]. Arsenic has a high affinity for sulfhydryl-containing glutathione. Binding of arsenic with sulfhydryl groups of glutathione[35] leads to the depletion of glutathione[36], resulting in inhibition of glutathione reductase, thus producing excessive reactive oxygen species in the testis. Increased production of free radicals leads to lipid peroxidation in the cell membranes and finally damage to the cell. Increased free radical production interferes with the functioning of the antioxidant defense system, and this results in tissue injury[37].

In the present study, it was observed that *M. pruriens* seed extract (500 mg/kg b.w., 1 000 mg/kg b.w.) protects the testis from arsenic toxicity. It was indicated by the increase in sperm count and a reduction in the number of abnormal sperms. A marked increase in sperm concentration, motility, and improved semen quality was seen in albino rats exposed to *M. pruriens*[38].

M. pruriens contain *L*-dihydroxyphenylalanine, which scavenges free radicals[39]. Hypothalamus is stimulated by *L*-dihydroxyphenylalanine to release gonadotropins, which in turn stimulate the anterior pituitary, resulting in the secretion of luteinizing hormone and follicle-stimulating hormone[39]. Elevated levels of these hormones stimulate the process of spermatogenesis *via* testosterone. Saponins reduce the formation of free radicals. The animals treated with extracts of *M. pruriens* have been shown to increase serum and testicular testosterone levels[40].

Mice treated with *M. pruriens* seed extract 2 000 mg/kg b.w. did not show any signs of toxicity or improvement concerning biochemical and sperm indices. Improvement in sperm indices and recovered levels of non-enzymatic antioxidants with *M. pruriens* 1 000 mg/kg b.w. along with sodium arsenite treatment were comparable to that of *M. pruriens* + *N*-acetylcysteine treated group.

Even though increased arsenic levels in drinking water (above 50 μg/L) have been reported in many countries like Nepal, Argentina, China, Japan, and Vietnam, the main stirred areas with

respect to extent of the population affected by the contamination and elevated exposure, are situated in Bangladesh and West Bengal. The arsenic concentrations in groundwater in these areas have been documented up to 3 200 μg/L[41]. Since arsenic-rich drinking water is inevitable in these areas, the susceptibility of testicular toxicity further leading to infertility or abnormal spermatogenesis resulting in possible teratogenesis is also unavoidable. Arsenic is also known to cause other tissue toxicity. Handling of toxicity is to be done in such a way that the body should not be burdened further. In this context, plant-based treatment may help, as the body can easily handle them without side effects. Further, *M. pruriens* is proved to have nutritional value and medicinal value[42]. Therefore, we can administer *M. pruriens* in the diet of arsenic insulted people so that both the nutritional and medicinal needs are taken care of.

In conclusion, administration of *M. pruriens* with sodium arsenite ameliorates the oxidative stress, reduces testicular tissue damage, and improves sperm morphology in mice. Additionally, *M. pruriens* reduces lipid peroxidation, significantly indicating its protective effect against the deleterious effects of arsenic on lipids. Thus, the beneficial effects of *M. pruriens* observed in testicular functions might have been due to the reduction of oxidative stress induced by the arsenic.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Authors' contributions

All the authors contributed significantly to the intellectual content, the analysis of data and the reviewing of final version of the work. Preethi Concessao and Laxminarayana Kurady Bairy designed the study; Preethi Concessao performed the research; Preethi Concessao and Laxminarayana Kurady Bairy analyzed the data; Preethi Concessao wrote the paper; and Preethi Concessao, Laxminarayana Kurady Bairy and Archana Parampalli Raghavendra revised and finalized the manuscript for submission.

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