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Molybdenum-Induced Oxidative Stress and Histopathological Alterations in the Epididymis

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Abstract

Molybednum is one of the trace elements required for proper functioning of the human body. The present study was designed to assess the effects of molybdenum on accessory reproductive organ i.e. epididymis. Male Wistar rats were administered with three different doses of molybdenum (50, 100 and 150 mg/kg body weight) for 60 days. A recovery study was also conducted in the highest dose group for which half the animals of the group were left untreated for the next 60 days. Exposure to molybdenum induced a significant decline in superoxide dismutase, ascorbic acid and glutathione while lipid peroxidation revealed a significant increase in epididymis in dose-dependent manner. Significant reduction in epididymal epithelial cells height, short sterocilia, increased intertubular stroma, decreased epididymal sperm reserve in the lumen and reduction in the diameter of cauda epididymal tubules with a relatively large amount of intertubular stroma was observed in epididymis of mice treated with molybdenum which might be correlated with enhanced oxidative stress. In the recovery group, significant improvement in antioxidant parameters and histoarchitecture of epididymis was observed indicating toxic effects of molybdenum can be reversed by cessation of exposure.

Keywords: Antioxidants, Epididymis, Histology, Molybdenum, Rats

1. Introduction

Male reproductive performance is dependent on complicated interactions of endogenous hormones and other growth factors. Disruption of this endogenous physiological milieu by the interaction of exogenous chemicals may have serious effects on reproductive organs as well as on the development of reproductive capacity and sexual behavior[1]. The male reproductive system is highly susceptible to toxic exposure throughout life. The reproductive system is highly vulnerable to characteristics of an individual including physical, biological, behavioral, cultural and socio-economic factors[2].

Augmented rate of industrialization, economic development and urbanization have led to drastic changes in the lifestyle of humans that have resulted in the extensive production and use of beneficial substances. Consequently, many perilous chemicals are being released into the environment at an alarming rate which might cause adverse impacts on both humans and wildlife[3].

Metals are everywhere in food, dietary supplements, water, air, alcoholic drinks and tobacco. Unlike organic pollutants, metals cannot be destroyed easily and accumulate throughout the food chain, producing potential health risks and ecological disturbances[4]. Some metals such as iron, zinc, manganese, copper, and

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molybdenum etc. is essential for good health. These are essential elements as cofactors in enzymes or act as a constituent of many chemicals in commerce and pharmaceuticals. Among them, molybdenum is one of the trace metals that are required as a cofactor for the functioning of various enzymes that promote oxidation of pyrimidines, purines and pteridines; catalyze the conversion of hypoxanthine and xanthine to uric acid and sulfite to sulfate. It is also required in the detoxification of N-hydroxylated compounds which can cause cancer[5]. It is being widely used in industries as well as in daily life. A few studies are available showing the toxicological effects of molybdenum on various systems in different animal models but the effects of molybdenum on epididymal tissue have been studied least to date. Therefore, molybdenum has been selected in the present study to gain knowledge about the possible effects of molybdenum exposure on male fertility by emphasizing on epididymal histology.

2. Material and Methods

2.1 Chemicals

Ammonium molybdate ($(NH_4)_6$ $Mo_7O_{24}.4H_2O$) was acquired from Merck Limited, Mumbai, India.

2.2 Experimental Animals

Male Wistar albino rats were purchased from IVARI Izzatnagar, Bareilly (U.P.), and India. These animals were used to develop a colony and the subsequent offspring were used for experiments. The rats were kept in polypropylene cages under standard laboratory conditions and provided with pallet diet and water *ad libitum*. The guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals were used during maintenance and use of experimental animals. Animal Ethical Committee of the Department of Zoology, University of Rajasthan, Jaipur approved the study (1678/GO/a/12/CPCSEA; 09.01.2013).

2.3 Experiment Design

40 Male rats were taken and divided into four groups each having eight rats except Group IV, which have 16 rats. Group I rat were treated with vehicle (distilled water, 0.5 ml/rat/day) for 60 days and considered as control rats. Group II, III IV were orally treated with ammonium molybdate (50,100 and 150 mg/kg body weight/day respectively for 60 days). For recovery study rats were left untreated for next 60 days after treatment withdrawal in the highest dose treatment group.

After completion of each group, overnight fasted rats were autopsied under ether anesthesia. Epididymides were taken out, adherent fat and blood clot was cleaned off and it was weighted. Half of the epididymis tissue was fixed in Bouin's fluid for histopathological observations. The remaining half were kept frozen at -200 \-700 C for biochemical estimations. Lipid peroxidation (TBARs[6], Superoxide dismutase[7], Glutathione[8] and Ascorbic acid)[9] were analyzed in cauda epididymis.

2.3.1 Determination in Lipid peroxidation

The level of Malondialdehyde (MDA) in the testis was determined following the method outlined by Ohkawa *et al*[6]. The TBARS (Thiobarbituric Acid Reactive Substances) level was quantified as nanomoles of MDA per milligram of tissue. In brief, testicular tissue was homogenized in 1.15% KCl solution (10% w/v). Then, a portion of the tissue homogenate (0.1 ml) was mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (adjusted to pH 3.5 with NaOH), and 1.5 ml of 0.8% aqueous solution of Thiobarbituric Acid (TBA). The resulting mixture was vigorously mixed and incubated at 60°C for 60 minutes. After cooling, the mixture was extracted with 5 ml of a n-butanol and pyridine mixture (15:1, v/v). Following centrifugation at 4000 rpm for 10 minutes, the organic phase was analyzed spectrophotometrically at 532 nm.

2.3.2 Determination of Superoxide Dismutase

The activity of superoxide dismutase was assessed following the method described by Marklund and Marklund[7]. A solution containing 2.85 grams of Tris and 1.11 grams of EDTA-Na_2 dissolved in 1 liter of distilled water was prepared. Additionally, 0.252 grams of pyrogallol was dissolved in a solution composed of 0.6 milliliters of concentrated hydrochloric acid diluted in 1 liter of distilled water. The spectrophotometer was calibrated to zero using the Tris-EDTA buffer. Test tubes for both control and sample were prepared and then filled accordingly. The absorbance was measured at 420 nm against the Tris-EDTA buffer at zero time and after 1 minute from the addition of pyrogallol.

2.3.3 Determination of Ascorbic acid

To 0.5 milliliters of the sample, 1.5 milliliters of 6% Trichloroacetic Acid (TCA) was added and left to stand for 5 minutes before centrifugation. The supernatant was then mixed with 0.3 grams of acid-washed not and vigorously shaken, followed by filtration. This process converts ascorbic acid to dehydroascorbic acid. Subsequently, 0.5 milliliters of the filtrate were combined with 0.5 milliliters of DNPH, then stopped and incubated in a water bath at 37°C for precisely 3 hours. Afterward, it was removed, cooled in ice-cold water, and slowly mixed with 2.5 milliliters of 85% sulfuric acid. The tube contents were thoroughly mixed and left at room temperature for 30 minutes. A series of standards containing 20-100 micrograms of ascorbic acid were treated similarly, alongside a blank consisting of 2.0 milliliters of 4% TCA. The resulting color was measured spectrophotometrically at 540 nm.

2.3.4 Determination of Glutathione

Glutathione content was determined by Moron *et al*[8]. In short, an equal volume of 4% sulfosalicylic acid was combined with a portion of testis homogenate, and the resulting mixture was subjected to centrifugation at 10,000 g for 15 minutes at 4°C. Following this, 500 micro liters of the supernatant were mixed with 50 micro liters of 2, 2'-Dinitro-5, 5'-dithiobenzoic acid (DTNB). The level of GSH was directly proportional to the absorbance recorded at 412 nanometers. The results are presented in nanomoles per gram (n mol/g) of tissue.

2.3.5 Histopathology

Histopathology of cauda epididymis of control and treated rats was analysed and the mean height of cauda epithelial cells was also measured using oculometry.

2.3.6 Statistical Analysis

All the data except body weight were statistically analyzed with one way ANOVA using 10.0 computer software packages for windows (SPSS INC., Chicago, IL, USA) followed by LSD multiple comparison procedure as a post hoc test. An alpha probability of less than 5% ($p<0.05$) was calculated considerably significant.

3. Results

The rats treated with ammonium molybdate revealed a significant ($P<0.05$, $P<0.01$, $P<0.001$) decline in the relative weight of epididymides at 50, 100 and 150 mg/kg b.wt./day doses respectively. Although a significant improvement ($P<0.001$) was observed in the relative weight of epididymides after 60 days of dose withdrawal in the recovery group (Table 1).

The concentration of Thiobarbituric Acid Reactive substances (TBARs) represented a significant dose-dependent decline in epididymis of rats after treatment

Table 1. Relative epididymis weights and epididymal epithelial cell height of rats treated with various doses of ammonium molybdate

Treatments	Group-I Control (vehicle)	Group-II Ammonium molybdate (50mg/kg b.wt./day)	Group-III Ammonium molybdate (100 mg/kg b.wt./day)	Group-IV Ammonium molybdate (150 mg/kg b.wt./day)	Recovery-Group (after 60 days of treatment withdrawal)
Epididymides (mg/100 g b.wt.)	536.68 ± 13.89	528.39 ± 12.94 [a]	492.74 ± 14.75 [b]	419.45 ± 13.62 [c]	517.24 ± 13.43 [a***]
Cauda epithelial cell height (µ m)	24.75 ± 0.86	22.57 ± 0.68 [a]	20.75 ± 0.75 [b]	17.12 ± 0.72 [c]	21.87 ± 0.85 [a***]

Levels of significance: Values in parenthesis indicate % changes Values represent mean ± SEM (n = 8)

ns- Nonsignificant; a- P<0.05; b- P<0.01; c- P<0.001, final body weight compared with initial body weight.

ns- Nonsignificant; a- P<0.05; b- P<0.01; c- P<0.001, ammonium molybdate treated groups compared with control group.

+- nonsignificant; *- P<0.05; **- P<0.01; ***- P<0.001, recovery group compared with group-IV. (One way ANOVA followed by LSD multiple comparison tests)

with ammonium molybdate in all the treated groups, when compared with the control group. The increased rate of lipid peroxidation (TBARs level) was significantly (P<0.01) lowered in the epididymis of rats of the recovery group as compared to rats of Group IV. However, it was still high (P<0.05) when compared to control values (Table 2). The activity of Superoxide Dismutase (SOD), glutathione content and ascorbic acid concentration in epididymis was found to be significantly (P<0.05, P<0.01, P<0.001) dose-dependent decline in rats treated with various doses of ammonium molybdate (50, 100 and 150 mg/ kg b.wt/ day; respectively). However, significant improvement was noticed in the Superoxide Dismutase (SOD) activity (P<0.01), glutathione concentration (P<0.01) and ascorbic acid (P<0.01) in epididymis after 60 days of treatment withdrawal when compared with Group-IV (150 mg/kg b.wt./day) rats (Table 2). A significant decline was noted in epithelial cell height of cauda epithelium in rats treated with ammonium molybdate (P<0.05, P<0.01, P<0.001; respectively) in dose dependent manner. In recovery group, height of cauda epithelial cell height was significantly improved (P<0.001) as compared to rats of Group IV (150 mg/kg b.wt./day) but it was still low (P<0.05) when compared to control rats (Table 1).

Light microscopic examination of the H and E-stained sections of the control rat cauda epididymis showed normal histoarchitecture of the tubules. All the tubules were lined by pseudo-stratified columnar epithelium composed mainly of principal and basal cells. Principal cells were topped at their apical border with numerous long stereocilia bordering the lumen. The basal cells were scattered in a smaller number between the bases of the principal cells, lying on the basement membrane. The lumen appears to be full of spermatozoa. The tubules were separated by a small amount of peritubular vascular connective tissue (Figure 1(a) and Figure 1(b)). The histoarchitecture of the cauda epididymis of rats treated with ammonium molybdate (50 mg/kg b.wt./day) showed slight degenerative changes. There was a slight reduction in the tubular size and epithelial cell height as compared to the control group. The number of spermatozoa in the lumen also declined. Some cellular debris was present in the lumen (Figures 2(a), 2(b); Table 1). In 100 mg/ kg b.wt group of ammonium molybdate treated rats, the histological picture of cauda epididymis was characterized by a significant reduction in epididymal epithelial cells height, short sterocilia, increased intertubular stroma and decreased epididymal sperm reserve in lumen (Figures 3(a), 3(b); Table 1). There were further alterations in the histoarchitecture of cauda epididymis in rats treated with high doses of ammonium molybdate (150 mg/kg b.wt./day). The luminal epithelium showed reduction in cell height and a reduced number and size of sterocilia. Reduction in the diameter of cauda epididymal tubules with a relatively large amount of intertubular stroma was observed between the tubules. The lumen contained a scanty number of spermatozoa (Figures 4(a), 4(b); Table 1). After 60 days of treatment withdrawal in

Table 2. Lipid peroxidation and antioxidant defense system markers in epididymis of rats treated with various doses of ammonium molybdate

Treatments	Lipid peroxidation (TBARs) (n mol MDA/mg tissue)	SOD (unit/mgprotein)	Glutathione (n mol/g tissue)	Ascorbic acid (mg/g tissue)
Group-I **Control** (vehicle)	1.71 ± 0.09	8.19 ± 0.21	2.98 ± 0.10	1.42 ± 0.05
Group-II **AM** (50 mg/kg b.wt./day)	1.98 ± 0.11 [a]	7.48 ± 0.21 [a]	2.67 ± 0.07 [a]	1.21 ± 0.06 [a]
Group-III **AM** (100 mg/kg b.wt./day)	2.15 ± 0.11 [b]	7.13 ± 0.24 [b]	2.51± 0.10 [b]	1.09 ± 0.06 [b]
Group-IV **AM** (150 mg/kg b.wt./day)	2.52 ± 0.12 [c]	6.49 ± 0.17 [c]	2.05 ± 0.11 [c]	0.87 ± 0.04 [c]
Recovery-Group	2.05 ± 0.06 [a**]	7.44 ± 0.23 [a**]	2.58 ± 0.09 [a**]	1.19 ± 0.07 [a**]

Levels of significance: Values represent mean ± SEM (n=8)

ns- Nonsignificant; a- P<0.05; b- P<0.01; c- P<0.001, ammonium molybdate treated groups compared with control group.

+- nonsignificant; *- P<0.05; **- P<0.01; ***- P<0.001, recovery group compared with group-IV. (One way ANOVA followed by LSD multiple comparison test)

ammonium molybdate (150 mg/kg b.wt./day) exposed rats, the cauda epididymal histoarchitecture appeared near normal as evidenced by the increase in epithelial cell height, spermatozoa reserve in lumen and reduction in intertubular stroma (Figures 5(a), 5(b); Table 1).

4. Discussion

Reproductive organs are one of the important sensitive organs to toxic substances. Significant changes in absolute or relative weight of testis and accessory sex organ weights

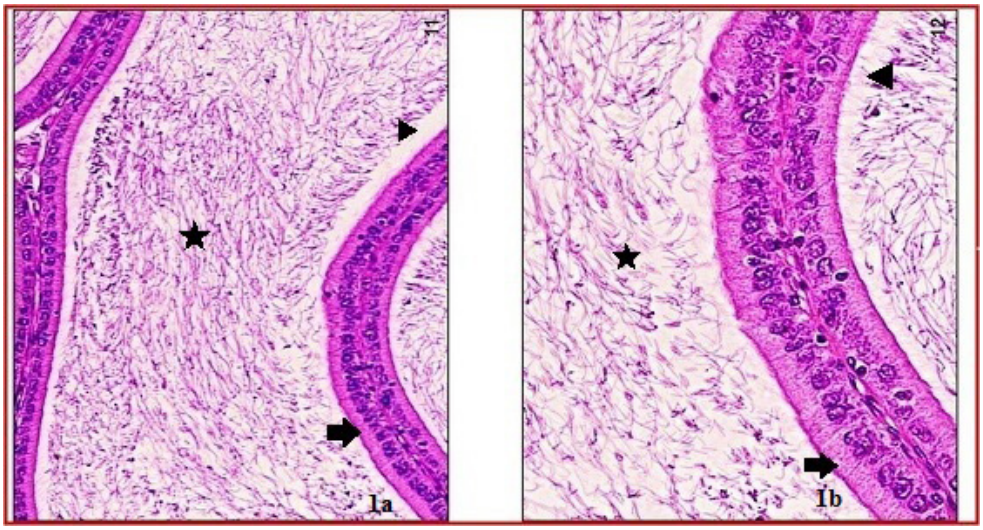


Figure 1(a) Photomicrograph of the cross section of cauda epididymis of control rat showing normal histoarchitecture (H.E. X 200).

Figure 1(b) Photomicrograph of the same at higher magnification characterized by closely packed tubules lined with low columnar cells with prominent nucleus, bearing stereocilia (★) and a large number of densely packed spermatozoa (▲) in the lumen. (H.E. X 400).

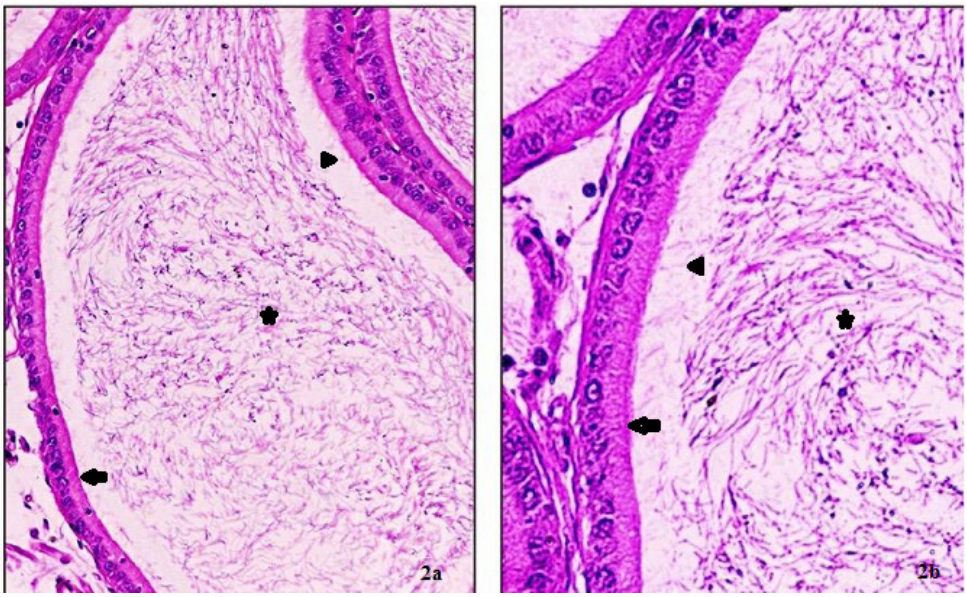


Figure 2(a) Photomicrograph of the cross section of cauda epididymis of ammonium molybdate (50 mg/kg b.wt./day) treated rat showing mild degenerative changes. (H.E. X 200).

Figure 2(b) Photomicrograph of the same at higher magnification. Note almost normal epididymal epithelium without any degeneration but epididymal cell height (➡) and sperm number (★) in lumen seems to be slighty reduced (H.E. X 400).

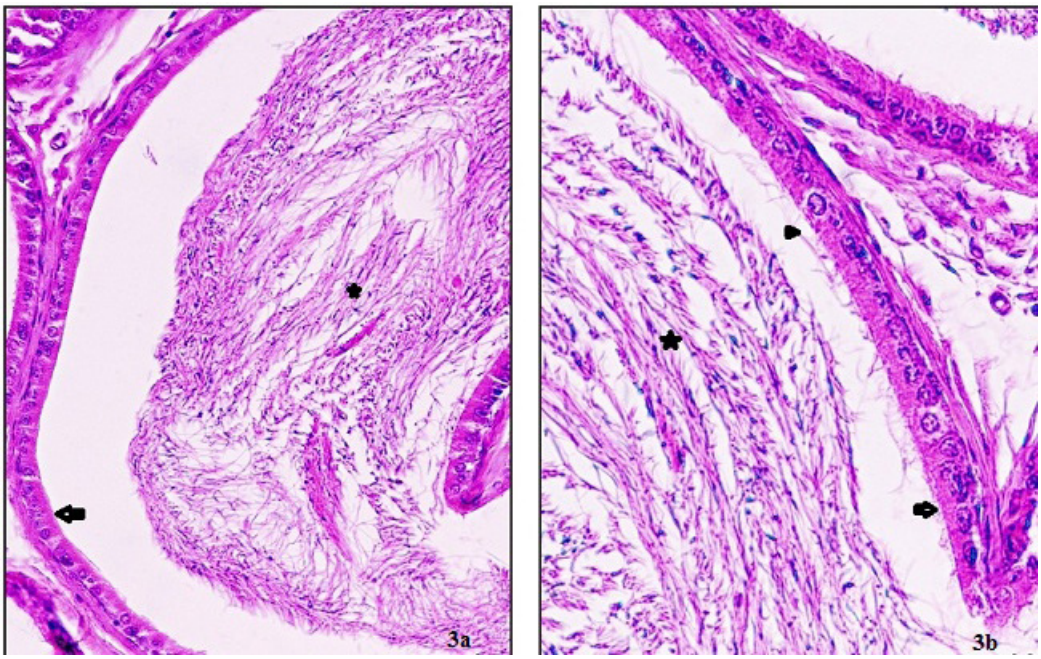


Figure 3(a) Photomicrograph of the cross section of cauda epididymis of ammonium molybdate (100 mg/kg b.wt./day) treated rat showing degenerative changes and decline in spermatozoa numbers (★) in tubular lumen. (H.E. X 200).
Figure 3(b) Photomicrograph of the same at higher magnification representing decreased epididymal sperm reserve (★) in lumen, decreased epithelial cell height (➡) presence of scanty short stereocilia (▲) and increase in intertubular connective tissue. (H.E. X 400).

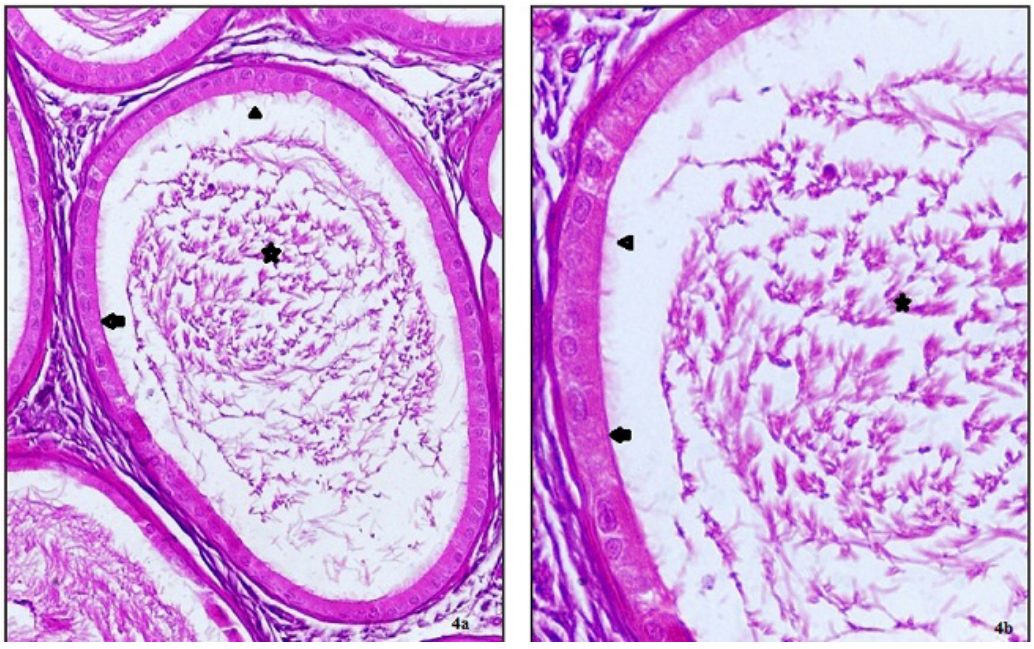


Figure 4(a) Photomicrograph of the cross section of cauda epididymis of ammonium molybdate (150 mg/kg b.wt./day) treated rat showing marked degenerative and atrophic changes in the epithelium (➡). (H.E. X200).
Figure 4(b) Photomicrograph of the same at higher magnification characterized by increased intra tubular space, cytoplasmic vacuolization in epithelial cells, reduced number of stereocilia (▲) and scanty number of spermatozoa in lumen (★) (H.E.400).

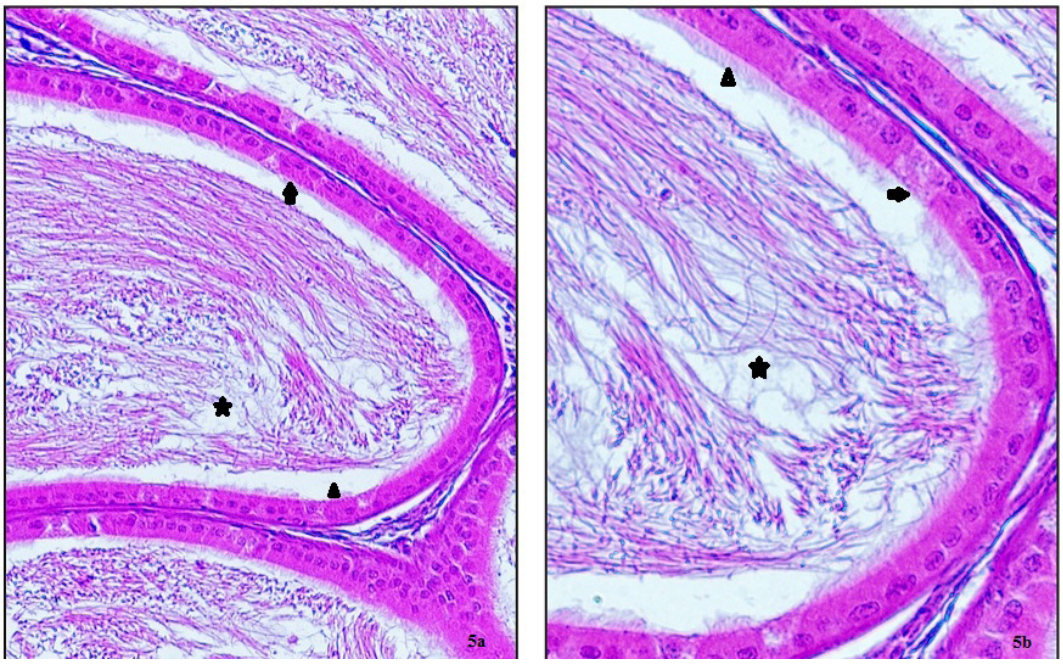


Figure 5(a) Photomicrograph of cauda epididymis of rat after 60 days of withdrawal of ammonium molybdate treatment (150 mg/kg b.wt./day for 60 days) showing epididymal tubules with normal histoarchitecture. (H.E. X 200).
Figure 5(b) Photomicrograph of the same at higher magnification exhibiting increase in epithelial cell height (➡) with prominent nucleus, presence of stereocilia (▲) and a large numbers of spermatozoa in lumen. (H.E. X 400).

may be used as crucial benchmark for toxicological studies. The epididymis provides a microenvironment for the maturation of spermatozoa including their gaining of progressive motility and fertilizing ability. Reduced testosterone level, diminished tubular size or decreased number of spermatozoa might be responsible for the decrease in the weight of epididymis[10,11]. A Similar reduction in the weight of epididymis was observed in molybdate treated animals[12,13]. Testosterone plays a crucial role in integrity and maintenance of the architecture of the epididymis therefore it can be concluded that deficiency of testosterone might be responsible for marked reduction in epididymal cell height and alterations in histopathology of epididymis[14]. In one of our previous studies, a reduction in serum testosterone was observed in molybdenum-treated rats which supported the alterations in histology of cauda epididymis[15]. These results came in accordance with the findings that reported disorganized and vacuolized cauda epithelial cell lining along with reduced sperm density in the lumen of cauda epididymis in molybdate[13]. Enhanced oxidative stress may also be credited for deteriorating changes in epididymis.

Augmented level of lipid peroxidation in rats treated with ammonium molybdate could be correlated with increased production of free radicals or reduced content/activity of antioxidants in epididymis. Increased levels of lipid peroxidation might have resulted from reduced level of intratesticular testosterone which in turn increases peroxidative damage, disruption of spermatogenesis and an increase in germ cell apoptosis. A significant elevation in TBARs level of epididymis suggests enhanced oxidative stress, consequently resulting in decline in the number, motility, viability of spermatozoa leading to infertility[16,17]. Zhai *et al.*,[18] reported a similar elevation in the levels of Thiobarbituric Acid-Reactive substances (TBARs) in epididymis of molybdate treated rats. Decline of SOD enzyme in epididymis may be due to overconsumption during the breakdown of free radicals. GSH is probably the most important antioxidant present in cells as it can interact directly with certain ROS (hydroxyl radical) to detoxify them, as well as plays a central role in a wide range of cellular functions, including protection, detoxication, transport and metabolism. Intracellular GSH status is a sensitive indicator of the health of a cell and tissue[19]. The reduced level of GSH may be due to increased utilization in trapping free radicals generated due to toxic effect of these metals in testis and epididymis. According to Sasmal *et al.*,[20] high intake of molybdenum hinders ascorbic acid

metabolism which might result in declined reserves of ascorbic acid in rats.

Histopathological assessment of cauda epididymis provides significant information about toxicity of a compound on testicular events[21]. A dose dependent regressive change was observed in the histology of cauda epididymis of rats treated with ammonium molybdate. Degenerative changes were noted in the epithelium lining of the lumen, an increase in the intertubular connective tissue, diminution in the cauda epithelial cell height, decline in the length and number of stereocilia along with sperm debris and scanty spermatozoa in the lumen. These results came in accordance with the findings who reported disorganized and vacuolized cauda epithelial cell lining along with reduced sperm density in the lumen of cauda epididiymis in molybdate[22]. It is well established that testosterone plays a crucial role in integrity and maintenance of the architecture of epididymis. Therefore, these changes can be allied to insufficient androgen supply to the organ which was supported by our previous study.

5. Conclusions

Metal contamination is a serious growing concern at global, regional and local level, and influence the well-being of living organisms, including humans. During last few decades, exposure to metals has risen considerably due to their extensive usage in various industrial processes. Based on the results, it can be concluded that overexposure of molybdenum might lead to deteriorative changes in the histology and antioxidant status of epididymis which may lead to disturbance in sperm quality as cauda epididymis is the storage site for spermatozoa.

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