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Original Article

Postnatal Exposure to Sodium Arsenite ($NaAsO_2$) Induces Long Lasting Effects in Rat Testes

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ABSTRACT

Objective: The present study was undertaken to investigate the effects of early postnatal exposure to sodium arsenite ($NaAsO_2$) on rat testis. **Materials and Methods:** Wistar rat pups were administered aqueous solution of $NaAsO_2$, 1.5 mg/kg body weight (bw) (experimental) and distilled water (control), respectively, by intraperitoneal route (i.p.) from postnatal day (PND) 1 to 14. Testes were collected after 1, 7 and 36 days (at PND 15, 21 and 50) after the treatment period (PND1-14) from the animals and immersion fixed in Bouin's fluid followed by paraffin embedding. Seven micrometer thick serial sections were cut and stained with hematoxylin and eosin for light microscopic observations. At PND 50, morphological features of sperms and their counting was carried out besides processing the perfusion-fixed testes for electron microscopy (EM). **Results and Conclusions:** The observations revealed an altered morphology of the seminiferous tubules (ST) along with degeneration and dissociation of spermatogenic cells in the experimental animals at PND 15, 21 and 50. Also, increased number of sperms with abnormal morphology and decreased sperm count was noted in the experimental animals. These features together with electron microscopic observations of abnormal mitochondria and apoptotic nuclei of spermatogonia and spermatocytes could be indicative of long-lasting adverse effects on the rat testis induced by exposure to *As* during early postnatal period.

Key words: Arsenic, postnatal period, seminiferous tubules, sodium arsenite, spermatozoa, testis

INTRODUCTION

Arsenic (*As*), one of the major environmental contaminants with its ubiquitous distribution in air, water and soil,[1] ranks first on the Superfund list of hazardous substances.[2] A steady increase in the levels of environmental *As* and its associated deleterious effects on biological systems has emerged as a matter of global concern. Millions of people across the globe are on the verge of getting afflicted with *As* exposure. A number of studies carried out in people who are occupationally or environmentally exposed to *As* have reported detrimental effects on various organ systems such as cardiovascular, gastrointestinal, nervous, hepatobiliary, urinary, integumentary, reproductive etc.[3,4] Consumption of drinking water contaminated with *As* is a major source of exposure to *As* particularly in Southeast Asian countries,[5,6] where *As* in the bedrock or soil seeps easily into the surrounding ground water resulting in *As* levels in water above the permissible levels advocated by the WHO[7] (10 parts per billion). Also, the use of *As*-contaminated ground water for irrigation purposes[8] as well as its anthropogenic emissions from mining, smelting, agricultural sources (pesticides and herbicides) and industrial sources (semiconductor devices, glass

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manufacturing, wood preservatives) contribute towards local exposures.

The past decade has witnessed a steady decline in the male reproductive health with a marked increase in the population of subfertile males.[9] As such, exposure to various factors including environmental contaminants has been proposed as the triggers for inducing spermatogenic failure and male infertility. *As* has been reported to get selectively accumulated in the testes[10] and epididymis.[11] The developing testis forms one of the major targets for *As*-induced toxicity due to absence of blood testis barrier and its high arsenite methylation activity.[12] Seminiferous tubules (ST), the seat of production of functionally mature spermatozoa, exist as sex cords up to PND 4 (the time period till the germinal precursor cells remain in a mitotically quiescent stage), followed by initiation of spermatogenesis. The early postnatal period is critical for the developing reproductive system (rodents) as the sexual differentiation of the central nervous system and hypothalamic pituitary gonadal axis[13] along with fixed ratio of Sertoli cell to germ cell is determined during this period. Besides, imprinting of male sexual behavior and growth hormone secretion pattern is best characterized in rodents and is reported to be influenced by testicular androgen production in the early postnatal period.[14] Accordingly, postnatal exposures to various agents including *As* could influence the fertility potential during adulthood. The present study was designed accordingly to determine whether exposure of rats to $NaAsO_2$ during early postnatal period induces any detrimental effects on maturation of testes with context to various time intervals. The time periods for obtaining the testes such as PND 15, 21 and 50 were chosen as these correspond to the formation of blood testis barrier (BTB), weaning and attainment of puberty, respectively.

MATERIALS AND METHODS

Animals and experimental protocol

Pregnant Wistar rats (*Rattus norverigicus*) (gestation days 17–19) were procured from the Central Animal Facility (CAF) of the All India Institute of Medical Sciences (AIIMS) after obtaining ethical clearance (IEAC-443/08) from Institute Ethical Committee (IEC). The animals were housed under standard laboratory conditions of light (12-hour light-dark cycle), temperature 20 ± 2°C and humidity 55 ± 5% in CAF (AIIMS). All the principles and procedures laid down by the IEC were followed strictly for animal care. The animals were fed standard rodent diet (Golden Feeds, India) and drinking water *ad libitum* and checked daily (10 a.m. and 4 p.m.) to note if they had delivered. The day of delivery of pups was considered as postnatal day (PND) 0. The pups were randomly divided into the control (I) and the experimental (II) groups and further subdivided (n = 6/subgroup) according to the time period of obtaining the tissue [Figure 1]. A single daily dose (1.5 mg kg^{-1} body weight (bw) of $NaAsO_2$ (Loba Chemicals, India) was administered to pups of experimental group from PND 1–14 by intraperitoneal (i.p.) route with the help of Hamilton syringe.[15-17] The dosage of $NaAsO_2$ was based on WHO guidelines according to which LD50 of $NaAsO_2$ in rats is 10 mg kg^{-1} bw and ED (1/10th of LD) is 1 mg kg^{-1} bw.[18] 15 mg of $NaAsO_2$ was dissolved in 10 ml distilled water so that 1 microliter of this solution for 1 g bw ensured dosage of 1.5 mg kg^{-1} bw. The i.p. route was chosen to ensure the exact and the proper uptake of *As*. The control group received equivalent amount of distilled water by the same route. The animals were observed for general features of well being all along the experimental period and weighed daily. The animals were sacrificed (PND15, 21, 50) either by cervical dislocation or by perfusion fixation (4% paraformaldehyde) under light ether anesthesia. The testes were removed and their wet weight was noted (monopan balance-Sartorius). After gross examination, the fresh testes were immersion fixed in Bouin's fluid and processed for light microscopic observations whereas the perfusion fixed testes (PND 50) were immersed in Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde) and processed for electron microscopic observations.

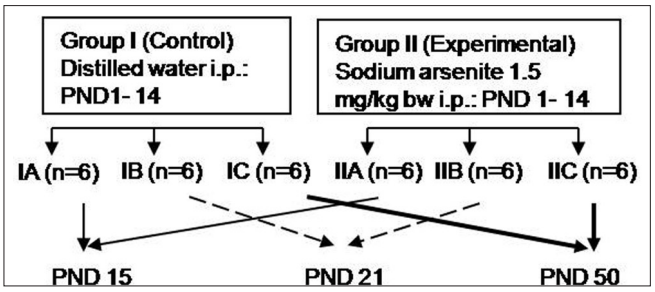


Figure 1: Animal groups (control and experimental) based on the substance administered and the day of obtaining tissue

Tissue processing (Light microscopy)

The fixed testes (Bouin's fluid) were processed for paraffin embedding and 7-μm thick serial sections were cut (rotary microtome, Shandon AS325) and stained with hematoxylin and eosin (H and E). Morphological and morphometric observations were carried out using Nikon E 600 microscope mounted with DS cooled camera and attached to Image analysis system software (NIS Elements, Advanced Research 2.3). For seminiferous tubule diameter (STD), luminal diameter (LD) and seminiferous epithelial height (SEH), four sections per animal were selected randomly from the mid portion of the testis and four randomly chosen areas per section were considered for measurements.[19] Only circular and nearly circular seminiferous tubules (ST) (minimum 100 tubules/testis) were taken into account.[20]

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Cavalieri estimator

From an exhaustive series of serial sections (7 μm), every 50th section was sampled for the estimation of volume (first section being randomly selected) and at least 10 sections were studied from each sample. A graticule with small squares of 4000 μm dimension was superimposed on the sections (2×). Only those intersections of the superimposed grid were counted whose top right corner was associated with point inside the object. Total number of points landing on all the lookup sections was used to calculate the volume of the testis: $V = T * a/p * \Sigma^{m}_{i=1} P_i$, where, V = volume of the testis, T = thickness of the slab = periodicity * section thickness, a/p (area per small square) = 16000000 μm^2, P_i = number of points landing within the object transect on the i^{th} section. [21]

Tissue processing (Electron microscopy)

The fixed testicular tissue (Karnovsky fluid) was further processed for transmission electron microscopy and the ultrathin sections (uranyl acetate and lead citrate stained) were observed under the TEM, 268D Morgagni, Philips (SAIF - Sophisticated Analytical Instruments Facility - DST, AIIMS).

Epididymal sperm morphology and count

The small fragments of cauda epididymis were minced in normal saline (1.0 ml) followed by homogenization in vortex shaker. Ten microliter of this suspension was added to an equal volume of eosin on a slide for smear preparation. Two hundred sperms/testis were scanned and classified according to regional abnormalities (head and tail); the results being expressed as percentage. Ten milliliter of the same suspension was also loaded on both the grids of the hemocytometer and the number of sperms in 5 squares/grid was counted (20×) and expressed in millions/ml.[22]

Statistical analysis

The data for various parameters was expressed as mean ± standard deviation (SD). Unpaired two-tailed *t* test was applied for comparison between the control and the experimental groups, using SSPS software. The *P* value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Body weight

Figure 2 shows the growth curves obtained from the daily recording of the bw of the control and the experimental animals during the treatment period (PND 1-14). A uniform pattern in the rate of gain in bw of the control and the exposed animals was observed till PND 6. However, from PND 10 onwards, a significant ($P < 0.05$) decrease in the rate of gain in bw of exposed animals was evident. Moreover, there was a significant ($P < 0.05$) decrease in the bw of the exposed animals at PND 15, 21 and 50 as compared to age-matched controls [Table 1]. The significant decrease ($P < 0.05$) in bw of exposed animals is in agreement with the earlier reports.[23,24] Rodriguez and coworkers (2002) observed decreased bw in young rats (exposed to *As* via drinking water from PND 1 **to** 4 months) and attributed it to the general weakness of the experimental animals making them lag behind in suckling (within litter competition). On the other hand, *As*-induced interference in the copper metabolism of adult rats has been

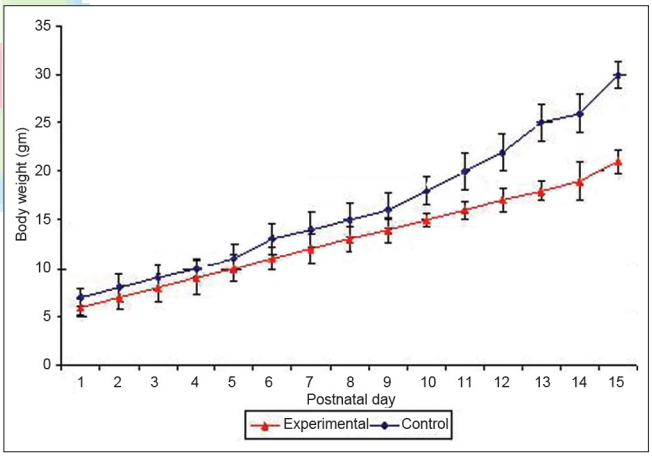


Figure 2: Effect of sodium arsenite exposure (PND 1-14) on the growth curves based on the daily recording of the body weight of Wistar rat pups during the treatment period

Table 1: Effect of sodium arsenite exposure (PND 1-14) on body weight, testis volume, testis weight, Seminiferous tubule diameter (STD), luminal diameter (LD), seminiferous epithelial height (SEH) of Wistar rats at PND 15, 21 and 50

Parameters	PND 15		PND 21		PND 50	
	Control	Expt.	Control	Expt.	Control	Expt.
Body wt (gm)	30 ± 1.41	21 ± 1.26*	46.5 ± 2.16	40 ± 1.41*	215.16 ± 9.28	138.33 ± 27.86*
Testis vol (mm^3)	720.62 ± 15.63	1910.6 ± 26.131*	1034.92 ± 11.72	1512.2 ± 13.12*	19613.05 ± 75.24	9541.82 ± 55.81*
Testis wt (gm)	0.053 ± 0.005	0.073 ± 0.01*	0.14 ± 0.02	0.126 ± 0.017	1.42 ± 0.30	0.756 ± 0.19*
STD (µm)	69.42 ± 1.42	73.95 ± 9.35	101.31 ± 7.91	106.82 ± 8.68	239.77 ± 1.91	237.45 ± 1.62
LD (µm)	-	-	24.45 ± 2.2	27.41 ± 2.94	105.133 ± 2.53	103.07 ± 1.84
SEH (µm)	-	-	34.58 ± 3.37	32.89 ± 2.82	52.42 ± 2.20	61.58 ± 3.81

The values are expressed as (Mean ± SD); *P* value < 0.05 is considered as significant (*)

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associated with decreased food intake resulting in decrease in the bw.[23] Other investigators,[22,25,26] in contrast, did not observe any significant difference in the bw of the adult animals exposed to *As* for varying periods.

Weight and volume of testes

The testicular weight exhibited a definitive pattern in the experimental animals with a significant increase ($P < 0.01$) at PND 15, weight comparable with the controls at PND 21 and a significant ($P < 0.05$) decrease in the testicular weight at PND 50 [Table 1]. Also, the mean volume of the testes presented a significant increase ($P = 0.029$) in *As*-exposed animals at PND 15 whereas a significant decrease ($P = 0.029$) was observed in testicular volume at PND 21 and 50 as compared to the controls [Table 1]. The increase in the testicular weight (wet) and volume in the exposed animals at PND 15 and a decrease at PND 50 could be indicative of an initial inflammatory response of testicular tissue (ST and Leydig cells) to *As* exposure followed by testicular atrophy resulting from prolonged exposure to accumulated *As*. These observations are consistent with the earlier reports.[16,22,26] Sarkar and coworkers (2003) observed a dose-dependent decrease in the testicular and accessory sex organ weight in adult Wistar rats following i.p. administration of $NaAsO_2$ (4, 5 or 6 mg/kg bw) and associated it with *As*-induced testicular atrophy.[22] Earlier studies have reported the preferential accumulation of heavy metals in the testes and epididymis as the underlying factors for long-lasting exposure.[10,11] A significant decrease in the weight and volume of testes (Wistar rats) was reported following $NaAsO_2$ exposure and/or vasectomy.[27] These investigators associated this observation with $NaAsO_2$-induced oxidative stress in the reproductive system leading to suppression of spermatogenesis. At the central level, high levels of *As* have been reported to suppress the sensitivity of gonadotrophs to GnRH as well as gonadotropin secretion by elevating the plasma levels of glucocorticoids, ultimately leading to gonadal toxicity and influencing the testicular volume.[28] Alternately, the role of FSH in inhibiting normal degeneration of germinal cells has been suggested,[22] so that *As*-induced reduction in FSH could be the underlying factor for reduction of the testicular volume.

Light microscopic observations

The testicular sections of control animals at PND 15, 21 and 50 presented round to oval profiles of ST having germinal cell lineage at different levels of maturation along with intervening interstitial tissue containing Leydig cells [Figures 3 a, c, e]. Lumen was evident in a few tubules as early as PND 15. The seminiferous epithelium of exposed testes showed graded disruption with disorganization and vacuolization of the epithelium at all the ages studied [Figures 3b, d, f]. $NaAsO_2$-induced structural alterations and loss of germinal cells in the ST could be traced to absence of blood testis barrier (BTB) during the treatment period

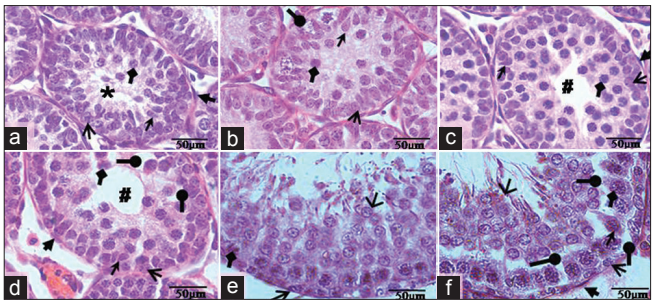


Figure 3: Morphological features of seminiferous tubules of rat testis at PND 15 (a, b); PND 21 (c, d); PND 50 (e, f) from control (a, c, e) and experimental (b, d, f) groups; ST show well-defined BL, with MC (➡), beginning of lumen formation (*) and Lumen present (#). SE cells are seen: Sp (➡), St (➡), Sc (➜), Sd (➔). SEH is decreased from 3-4 cell layers in (a) to 1-2 cell layers in (b); 5-6 layers in (c) to 3-4 layers in (d). Increase in SEH from 3-4 cell layer in (e) as compared to 5-6 layers in (f). Vacuolization (⬬) in b,d,f. (Scale bar 50 µm)

(BTB formation in rats – PND 15 to 17) in rats, thereby resulting in free access and accumulation of *As* in the ST. Spermatogenesis involves rounds of cellular multiplication (mitosis and meiosis) along with differentiation of the spermatids to spermatozoa, with a number of enzymes and cofactors being involved in these processes. *As* has been reported to target enzymes involved in spermatogenesis by binding to thiol groups of various proteins[29] and reducing the gene expression of key enzymes resulting in modulation of androgen receptors.[26] The decrease in the spermatogenic cell lineage has been associated with the ability of *As* to bind to tubulin, thereby destabilizing the microtubules and disintegrating the mitotic spindle, ultimately affecting the germ cell divisions, though the present data does not provide a direct link between exposure to $NaAsO_2$ and microtubular damage. Zheng and co-workers (2005) noticed evidence of apoptosis in germline cells, especially the spermatogonia and spermatocytes and suggested an association between *As*-induced cell death and conformational changes in Bax (pro apoptotic protein) with its subsequent translocation from cytosol to mitochondria resulting in activation of caspase-induced apoptosis.[30] Multinucleate cells, as observed in the lumen of a few ST in the present study, have been previously reported following treatment either with As alone[31] or combined exposure to As with fluoride[32] and have been linked to defective cytokinesis.

Morphometric analysis

Table 1 shows the values of STD, LD and SEH in the testes of animals exposed to $NaAsO_2$ (PND 1-14). The results show an apparent increase in STD and LD of ST in exposed group (PND 15 and 21) and an apparent decrease in these parameters at PND 50. As mentioned earlier, initial inflammatory response could be the underlying factor for increased STD (PND15) whereas testicular atrophy following exposure to accumulated *As* could present as decrease in the STD (PND 50). The optimum

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levels of testosterone are necessary for normal physiology and morphology of ST.[33] *As*-induced reduction in the levels of testosterone has been suggested as one of the underlying factors inducing shrinkage of ST.[16,33] A number of investigators have also reported decrease in the STD following exposure of adult rats to *As*.[22,27] Spermiation, the release of sperms into the lumen of ST, is the final step in spermatogenesis with tubulin playing a key role in the process.[34] With binding of *As* to tubulin,[35] the process of spermiation could get hampered, resulting in non release of sperms. In the present study, increase in SEH at PND 50 could be associated with non release of sperms. Thus, we propose that *As*-induced interference of various key molecules vital for spermatogenesis could alter the morphological and morphometric parameters of developing ST, these effects being long lasting and persisting up to puberty.

Sperm Parameters

A significant ($P < 0.0001$) decrease in the sperm count was observed in animals exposed to $NaAsO_2$ as compared to their age-matched controls [Table 2]. Microscopic observations of the sperm morphology revealed increased incidence of tail (34%) and head (6.5%) anomalies in the exposed group as compared to the controls [Figure 4]. Various parameters reflecting male infertility include low sperm count and motility as well as their poor quality.[22] Low sperm count has been associated with decreased production in the testes, their retention in the seminiferous epithelium, phagocytosis in the excurrent ducts or blockage in the excurrent ducts.[36] Significant decrease in the sperm count of exposed animals could be the result of depletion of germ cell lineage (supported by the observed apoptosis and vacuolization in the seminiferous tubules) and/or due to failure in the release of the differentiating spermatids into the lumen (based on increase in the SEH). Significant decrease in the number of spermatozoa after $NaAsO_2$ or oestradiol treatment in adult rats has been reported.[25] Evident increase in the sperms with tail defects (curved, bent, coiled) could be the result of protracted interaction of accumulated *As* with thiol and sulfydryl groups (abundant in the mammalian sperms).[10] Decreased sperm count and increased abnormal sperms have also been reported in adult mice following *As* exposure in isolation[10] or exposure to *As* in combination with sodium fluoride.[32] *As*-induced oxidative stress has been linked to cellular toxicity[37] and the increased vulnerability of spermatozoa to *As*-induced oxidative stress has been traced to their high polyunsaturated fatty acid content and relatively low activity of antioxidant enzymes.[38] Thus, it could be presumed that *As*-induced oxidative stress and inadequate protein turnover adversely affects the cellular metabolism resulting in decreased production of testicular enzymes and testosterone, in turn getting reflected as deranged sperm morphology.

Table 2: Effect of sodium arsenite exposure (PND 1-14) on sperm count and sperm morphology (percentage of normal sperms, sperms with head and tail abnormality) in Wistar rats at PND 50

Parameter studied	Control group	Experimental group	*P* value
Sperm count (×10^6)	6.47 ± 0.14	4.39 ± 0.16	0.029*
% Normal Sperms	72.75 ± 1.5	61 ± 1.82	0.029*
% Head abnormality	5.5 ± 0.57	6.25 ± 0.95	0.343
% Tail abnormality	21.75 ± 1.70	32.5 ± 1.29	0.029*

The values are expressed as (Mean ± SD); *P* value < 0.05 is considered as significant (*)

Ultrastructural observations

The testes of control animals revealed normal organization of the seminiferous epithelium resting on intact multilaminar basal lamina enriched with myoid cells [Figure 5a] and presenting intact cell junctions. The spermatozoa were embedded in the Sertoli cells and round mitochondria with cristae were clearly seen in their cytoplasm [Figure 5b]. The mitochondria with vesicular cristae could be distinctly seen in seminiferous epithelial cells [Figure 5c]. The acrosome formation with accumulation of Golgi apparatus towards the apical part of the nucleus was evident within the spermatids [Figure 5d]. Sertoli cells were seen with elongated nuclei and surface indentations containing embedded heads of developing spermatozoa. However, features suggestive of disruption and degeneration of the seminiferous epithelium were evident in the testes of exposed animals and included dissociation of spermatogenic cells [Figure 6a], deformed mitochondria dispersed throughout the cytoplasm of germ cells [Figure 6b], swollen and disorganized network of SER aggregated near acrosome formation in spermatids [Figure 6c], irregularly clumped chromatin together with blebbing of nuclear membrane in spermatocyte nuclei [Figure 6d], the electron dense cytoplasm with increased number of lysosomes in the Sertoli cells [Figure 6e] and discontinuous cell membrane and electron dense areas within developing spermatozoa [Figure 6f].

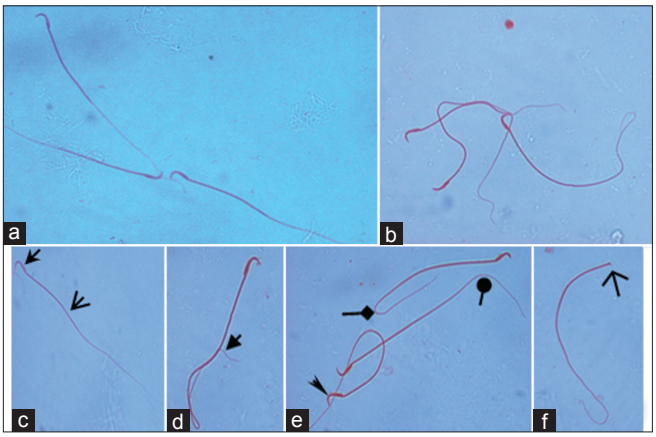


Figure 4: Low (a, b) and high (c, d, e, f) magnification photomicrographs of sperms from rat epididymis (PND 50) showing normal sperms with hook-shaped head (➞) and straight tail (➔) in control animals (a, c). Abnormal sperms with coiled tail (➡), bent tail (➡), curved tail (➡), head winding around tail (➡) and absence of head (➡) (d, e, f) from experimental animals. Scale bar 100 µm (a, b), 50 µm (c, d, e, f)

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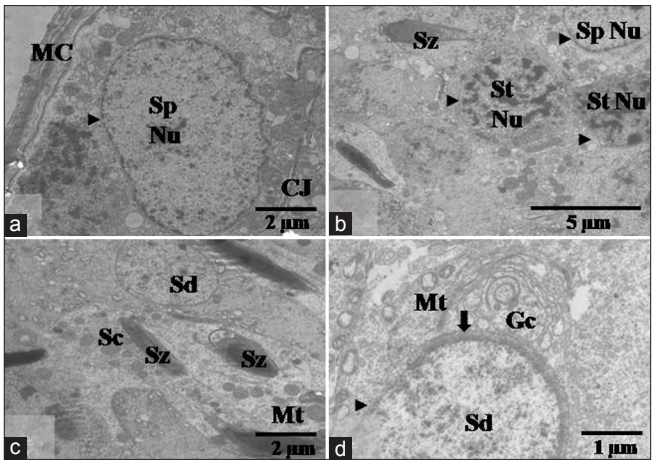


Figure 5: Photoelectron micrographs of control rat testis (PND 50) showing well-defined cell junction (CJ) between spermatogonia (Sp) and adjacent cell (a); mitotic bodies in primary spermatocyte nucleus (St nu) (b); Spermatozoa (Sz) embedded in the Sertoli cell (Sc) and round mitochondria with cristae (c); beginning of acrosome formation (⬇) within spermatid (Sd) and accumulation of Golgi apparatus (Gc) towards the apical part of the nucleus (d)

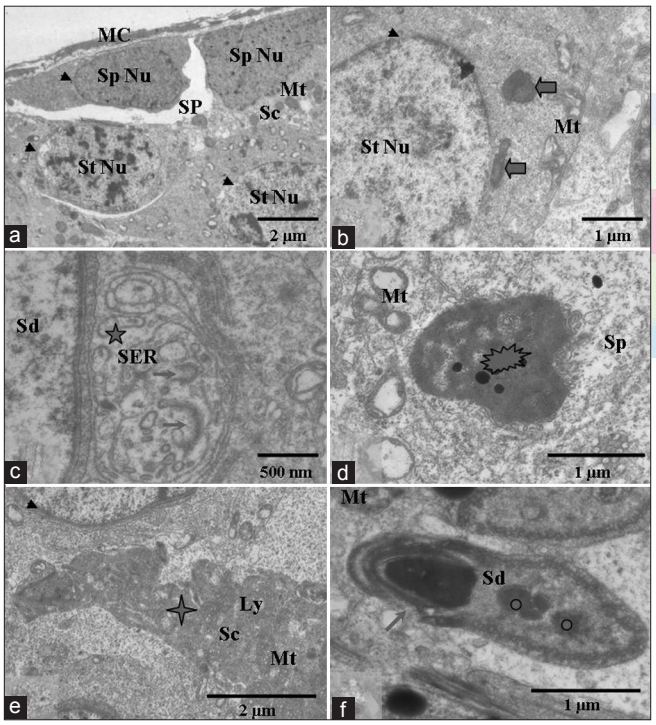


Figure 6: Photoelectron micrographs of experimental rat testis (PND 50) showing dissociation of spermatogenic cells (a), deformed mitochondria dispersed throughout the cytoplasm of germ cells (⬅) (b), swollen and disorganized network of SER (★) aggregated near acrosome formation in spermatids (➡) (c), St Nu undergoing apoptosis (➡) (d), the electron dense cytoplasm with increased number of lysosomes in the Sertoli cells (✹) (e) and discontinuous cell membrane (+) and electron dense areas within developing spermatozoa (○) (f)

The junctional integrity between the Sertoli cells, peritubular myoid cells, Leydig cells and germ cells plays a crucial role in complex and dynamic process of continued sperm production,[3] with spermatogonia and adjacent Sertoli cells constituting the nursery units for the developing sperms.[39] Faulty cell junctions and dissociations of the seminiferous epithelium in the exposed testes pointed towards disrupted intercellular communication. Similar observations regarding dissociations in the seminiferous epithelium have earlier been reported[40] following exposure to anabolic androgen steroid (nandrolone decanoate). The altered mitochondrial structure could be the aftermath of *As*-induced inactivation of mitochondrial enzymes and oxidative phosphorylation. Smooth endoplasmic reticulum (SER) with electron dense swollen vesicles spotted in certain spermatocytes could be due to *As*-induced affliction of lipid metabolism. Dilation of SER and Golgi apparatus has earlier been reported in rats following exposure to other substances such as aluminium.[41] The electron dense cytoplasm of the Sertoli cells along with a marked increase in their lysosomal number is considered as a marker of progressive degeneration. It has been further suggested that *As*-induced oxidative stress could compromise with the supportive role of Sertoli cells resulting in altered sperm parameters,[42] ultimately resulting in male infertility.

There is growing evidence that endocrine disruptors in the environment might be playing a substantial role in adversely influencing the reproductive system (semen quality is deteriorating by as much as 3% per year).[43] Potency of *As* as an endocrine disruptor has been reported with studies establishing alterations in Leydig cell structure and function following exposure to $NaAsO_2$.[16,44] The light and electron microscopic observations of the present study suggest increased vulnerability of the neonatal rat testis to $NaAsO_2$ exposure from PND 1 to 14 (critical window period), being evident as immediate adverse effects on testes (PND 15) as well as long-lasting spermatotoxic effects (PND 50) thereby pointing towards the association between *As*-induced structural alterations and resultant impaired reproductive function in later life. These speculations are further reinforced by the structural abnormalities observed both in the gonad as well as the gamete at PND 50 even though the exposure to $NaAsO_2$ extended only through PND 1-14.

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