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Subchronic cyanide toxicity on male reproductive system of albino rat

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Abstract

Sexually matured albino rats were orally treated with sodium cyanide, NaCN (0, 0.64, 1.2 and 3.2 mg/kg BW) for 90 days. After 90 days of treatment, the rats were euthanized and male reproductive functions were assessed by histopathology, sperm head counts, sperm motility, sperm morphology and hormonal assay. Only higher dose (3.2 mg/kg BW) of NaCN caused significant changes in body and reproductive organs weight, sperm motility, sperm count, sperm abnormality and in the levels of Luteinizing hormone (LH), Follicular stimulating hormone (FSH) and testosterone, whereas the group treated with 1.2 mg/kg BW showed significant changes in testis and prostate weight, sperm motility, sperm count, LH and testosterone levels. In contrast, insignificant changes were observed in body weight gain, reproductive organs weight, sperm parameters and hormonal levels in the rats treated with lowest (0.64mg/kg BW) dose of NaCN. Histopathologically, NaCN caused atrophy, degeneration in testis, increased number of clearing cells, vacuolation in epididymis and decrease secretion, desquamations of glandular epithelium in prostate were observed only at higher (1.2 and 3.2 mg/kg BW) dose levels compared to control. Whereas no changes in histology were observed in the rats treated with 0.64 mg/kg BW. Our results suggest that high (3.2 mg/kg BW) dose of NaCN can exert reproductive toxicity in male Wistar albino rats.

Keywords: Sodium cyanide, Reproductive toxicity, Histopathology, FSH, LH, Testosterone

Introduction

Cyanide is known as one of the most rapidly acting poison.[1] It is used extensively in industrial process such as electroplating, plastic, chemical synthesis and in mining operations through the milling of high grade ores and heap leaching of low grade ores throughout the world. [2-4] From all these industrial usage of cyanide, it has been released into the environment.[5] The estimated free cyanide in the industrial effluent was more than 14 million kg/year.[6] Consequently, the discharge of such cyanide contaminated industrial effluent may lead to the environmental pollution.[7] By this means of cyanide contamination, non-target animals like humans were exposed to the cyanide. Furthermore, animals and humans were exposed to the cyanide by the consumption of many plants, including bitter

almond, cassava, apricot etc, as a source of carbohydrates; contain cyanogenic glycosides that can release cyanide after metabolism.[8,9,10]

Apart from these two above mentioned rout of cyanide exposure, animals and humans were exposed to cyanide by cigarette smoke and smoke from industries containing the free cyanide, and by certain drugs, such as laetrile and nitroprusside also release cyanide ions after metabolism.[8,11-13] Rao *et al.,* [14] reported that globally, 26000 people were exposed to cyanogens every day; these compounds release toxic levels of cyanide ions in the body. By all these sources, it has created complex problems in modern society and in the environment.[1,15,16]

In fact, only free cyanide (i.e., the sum of molecular hydrogen cyanide, HCN, and the cyanide anion, CN^-) is considered to be a biologically meaningful expression of cyanide toxicity, regardless of its origin.[3] The toxicity results by inhibiting the enzyme, cytochrome *c* oxidase in the electron transport system of mitochondria and disrupting the aerobic production of adenosine triphosphate (ATP) and leads to anaerobic respiration.[17-20] However, with the help of another mitochondrial enzyme rhodanase, lower doses of cyanide can get detoxified from the body without causing any harm. The enzyme rhodanase helps to metabolize most of the absorbed cyanide to a cytotoxic metabolite, which is known as thiocyanate (SCN^-). Consequently, Bradbury *et al.,* [10] found higher level of thiocyanate (SCN^-) in school children consuming more cassava at the time of cassava harvest. But, the SCN^- has specific antithyroidal properties and its bioconcentration has been implicated as a possible etiologic factor in the alteration of thyroid function and development of goitre in humans and rats.[21,22]

Studies demonstrated that cyanide affect spermatogenesis via the hypothalamic-pituitary-gonadal axis in male rainbow trout.[23] Kamalu, [24] has observed cyanide can reduce spermatogenic cycle, testicular germ cell sloughing, degeneration and occasional abnormal cells in dogs. Whereas, several studies demonstrated that maternal consumption of cyanogenic plants lead to the fetal malformations in pigs, horses, sheep, cattle and humans.[8,25] But, studies pertaining to the cyanide toxicity on male reproductive system of Wistar albino rats were very rare. Two decades back in 1993 National Toxicology Program (NTP)[26] evaluated NaCN male reproductive toxicity in F344/N rat strain and found that up to100 ppm (4.5 mg/kg BW) of cyanide can cause mild (insignificant)

alteration in male reproductive system. However, several other studies demonstrated that cyanide (dose lower than the 4.5 gm/kg BW) can induce hepatotoxicity, renal toxicity, neurotoxicity, oxidative stress in functionally different tissue. [15,20,27,28] Therefore, based on the review of literature, the present study was executed to examine the hypothesis; sub-chronic exposure of cyanide may be affect male reproductive system in Wister albino rats.

Materials and methods

Chemicals

Sodium cyanide of 95% purity was procured from Loba Chemie Pvt. Ltd., Mumbai, India. Doses were freshly prepared by dissolving NaCN in double distilled water using standard volumetric flask.

Animals

Sexually matured (90 days old) male Wistar albino rats weighing about 180-190 g were utilized for the present study. Animals were maintained at the animal care facility in the Department of Zoology, Karnatak University, Dharwad, in plastic cages, fed a standard laboratory ration and watered *ad libitum*, and exposed to a 12 h light/dark cycle, under the controlled temperature (23 ± 2ºC) and air humidity of 65 ± 5 %. All animals were acclimatized for one week before the initiation of experiments and handled in accordance with the CPCSEA guidelines for the care and use of laboratory animals.

Experimental design

After the period of acclimation, animals were randomly divided into four groups of seven animals each and treated with respective doses.

Group I – Control animals (Received the vehicle only)

Group II – 0.64 mg/kg BW cyanide (this dose equals to $1/10^{th}$ of LD_{50})

Group III – 1.2 mg/kg BW cyanide (this dose equals to $1/5^{th}$ of LD_{50})

Group IV – 3.2 mg/kg BW cyanide (this dose equals to $1/2^{th}$ of LD_{50})

The selected LD_{50} value of the NaCN was based on available literature.[29] The treatment was given in the morning (between 09:00 and 10:00 h) to non-fasted rats for 90 days. The dose volume equals to 1 ml/ 100 gm BW and treated through oral gavage.

Clinical signs

Clinical signs and behavioral changes were observed daily in all groups for attraction to feed and water, activity or depression, responsiveness to tapping at the cage wall.

Body and reproductive organs weights

After 90 days treatment, all animals were sacrificed under light ether anaesthesia and taken final body weight on the electric balance. The weight of reproductive organs including testis, epididymis and prostate of respective groups were recorded after the scarification of animals.

Sperm motility

The epididymis was collected as quickly as possible and placed in clean petri plates. The cauda epididymis was cut into several pieces, and then incubated in 3 ml pre-warmed phosphate buffer saline (PBS) solution at 37ºC for 10 min to allow the sperm to release from the cauda epididymis. The sperm suspensions were evaluated for sperm motility, sperm head count and sperm morphology. The sperm suspension was pipetted several times; one drop of the suspension was placed on a slide, covered by a 22×22 mm coverslip. At last 10 microscopic fields were observed at 400X magnifications using a phase-contrast microscope (Olympus BX51, Tokyo, Japan). The sperm were categorized on the basis of their motility as ‘‘motile” or ‘‘immotile”. The results were recorded as the percentage of sperm motility.

Epididymal sperm count

The sperm head count was determined with a hemocytometer. A sample of 0.5 mL of the sperm suspension was diluted with 9.5 mL of sperm diluting solution (5 g $NaHCO_3$, 1 ml formalin (35%) and 25mg eosin per100 ml distilled water). Then, 10 µL of diluted sperm suspension was transferred to each counting chamber and then was allowed to stand for 5 min, and concentration of epididymal sperm was evaluated as millions of sperm cells per ml of suspension under 400 x magnifications using a phase contrast microscope (Olympus CH20*i*). Finally calculated according to the formula,

Sperm count = Total number of sperm in 5 squares X 50000 X 100 (Sperms/mL).

Epididymal sperm morphology

For sperm morphology one drop of the suspension was smeared on glass slides then air dried and stained with 1% Eosin Y. The morphological abnormalities of sperms were evaluated, from total of

two hundred sperm per animal by using the criteria of Nahas *et al.,* [30] and Mori *et al.,*[31] and the results were recorded as the percentage of abnormal sperm.

Hormone assays

Blood samples were collected by cardiac puncture technique under sodium pentobarbital anaesthesia (40 mg/kg) in dry glass centrifuge tubes. The blood was then allowed to stand for 10 min at room temperature to clot and centrifuged at 3000 rpm for 5 min. at 4ºC. The serum was then collected into separate vial and subsequently subjected for the assessment of LH, FSH and T levels determined by Fully Automated Bidirectionally Interfaced Chemi Luminescent Immuno Assay.

Histopathology

For histopathological examination, the testis, epididymis and prostate gland tissues were dissected and the tissue samples were fixed in Bouin's fluid for 24 h, processed by using a graded alcohol series, and embedded in paraffin wax. The paraffin blocks were cut into 5µm thick by using semi-automated microtome (LeicaRM 2255) and sections were stained with hematoxylin and Eosin (H&E) for light microscopic examination. The sections were evaluated for histopathological lesions in the testis, epididymis and prostate (Table 3) on the basis of arbitrary scores (-/+). For each slide in every case at least 10 fields were randomly selected for such scoring and then a cumulative figure was obtained for each treatment group. Photographed, by using a phase contrast microscope (Olympus BX51, Tokyo, Japan) with an attached photograph machine (ProgResC3, Jenoptic-Germany).

Statistical analysis

The data were analyzed by using SPSS 16.0 for Windows and expressed as the mean±SEM. The significance was performed using one-way ANOVA followed by Tukey's post-doc or student's *t*-test. All statistical analysis was conducted at the significance level of $P < 0.05$.

Results

Clinical evaluations, body and reproductive organs weights

Death was not observed in all the treated and control groups throughout the experiment. There were clinical signs of toxicity observed in the behavioral activity, roaming, arrogant posture was found only in highest dose of cyanide (data not shown). Body weight gain and absolute weight of reproductive

organs did not change significantly ($P > 0.05$) at 0.64 and 1.2 mg/kg BW, except testis and prostate weight at 1.2 mg/kg BW. But the body weight gain and absolute weight of testis, epididymis and prostate gland was significantly decreased ($P < 0.05$) in rats treated with 3.2mg/kg BW of NaCN and prostate weight at 1.2 mg/kg BW compared to the control group (Table 1).

Sperm motility, sperm head counts and morphology

There was no significant ($P > 0.05$) difference was observed in the sperm motility, sperm head count and sperm morphological abnormality in the second group rats treated with 0.64 mg/kg BW. While, in the third group treated with 1.2 mg/kg BW shown significant ($P<0.05$) changes in the sperm motility and sperm count but, insignificant ($P > 0.05$) changes in the sperm morphological abnormality compared to control. However in the fourth group rats treated with 3.2mg/kg BW NaCN showed significant ($P<0.05$) changes in all sperm parameters including motility, count and abnormality compared to the control group (Table 2).

Hormone concentration

There was no significant ($P > 0.05$) differences were observed in serum level of the LH, FSH and T in the second group rats treated with 0.64 mg/kg BW NaCN. While in the third group rats treated with 1.2 mg/kg BW showed significant ($P<0.05$) changes in serum LH and testosterone level and insignificant changes were observed in the FSH level. However in the fourth group significant changes were observed in the serum FSH ($P < 0.05$) and LH and testosterone ($P < 0.01$) levels were observed compared to control (Fig. 1).

Gross histopathology

Testis

In the control group, normal testis histology with regular seminiferous tubules and spermatogenic cell lines with abundance of spermatids in the seminiferous tubules were observed (Fig. 2A). The second group treated with 0.64mg/kg BW showed no changes in the histoarchitecture of testis compared to the control (Fig. 2B). Third and fourth group rats treated with 1.2 and 3.2 mg/kg BW of NaCN respectively showed histological alteration, including atrophy, degenerated seminiferous tubules with cell debris in the lumina. In addition to this there was a thin population of spermatogenic cells,

spermatocytes, spermatids and spermatozoa in the tubules (Fig. 2C,D). All these changes are more prominent in the fourth group rats compared to the third group (Table 3).

Epididymis

In the control group, epididymal histology with luminal cell lines with abundant number of sperm was observed (Fig. 3A). In the second (0.64 mg/kg BW) groups not observable difference was seen compared to the control (Fig. 3B). In the third and fourth group, increase in the number of clearing cells with a vacuolation in laminar cell layer was observed. Addition to this low sperm density were observed be decreased compared to the control (Fig. 3C,D). These alterations were moderate in the fourth group and mild in the third group compared to control (Table 3).

Prostate gland

In the control group, normal prostate histology with normal luminal cell lines with abundant amount of prostate secretion was observed (Fig. 4A). Second group rats treated with 0.64 mg/kg BW shown normal histoarchitecture as in control prostate (Fig 4B). However, third and fourth groups treated with 1.2 and 3.2 mg/kg BW respectively show decreased prostate secretion in the lumen (Fig. 4C,D). And desquamations of glandular epithelium were only observed at 3.2 mg/kg BW (Fig.4D).

Discussion

The gold mines and cyanide using industries brought with them not only development, employment and wealth, but also the most overwhelming changes in the nature such as pollution, negative health impacts and ecological destruction.[32] Clark and Hothem, [33] has reported that, the industrial effluent and metal processing pound were containing free cyanide along with metallocyanide complex concentration ranging from 0.3 to 216 ppm and 200 to 300 ppm respectively. Thus, in the present study the first two chosen doses (0.64 and 1.2 mg/kg BW) are considered to be environmentally relevant.[34] However, the labourers working in these industries have every chance of cyanide exposure. Furthermore, occupational and dietary exposure to cyanide occurs by the large scale cassava processing and ingestion of cassava based food products.[35,36] In view of this, sub-chronic (90 days) effect of cyanide (NaCN) toxicity at sublethal doses (0.64; 1.2 and 3.2 mg/kg BW) were evaluated on male reproductive system of the albino rat.

This study was strongly supported by NTP study on NaCN.[26] However, the difference was, use of rat strain and selected dose levels. Wister strain rats were used in present study while, F344/N strain in NTP study. The higher dose (3.2 mg/kg BW) tested in the present study was lower than the dose (100 ppm/4.5 mg/kg BW) used in the NTP study. We evaluated hormonal levels, as they play very important substantiation in male reproductive toxicity assessment. The results of the present study reveal that Wister strain male albino rats were more susceptible to the cyanide ions and male reproductive organs may be having low cyanide detoxification capacity. These findings were agreed with Kimani *et al.,*[20] as these have demonstrated that cyanide detoxification mechanism varies from species to species and tissue to tissue.

The lower dose (0.64mg/kg BW) in the present study induced no significant ($P > 0.05$) alteration in body weight gain, reproductive organs weight. However, in the third group rats treated with 1.2 mg/kg BW showed significant reduction only in the prostate weight compared to control (Table 1). While in the fourth group treated with 3.2 mg/kg BW showed significant ($P < 0.05$) changes in body weight gain, reproductive organ weights compared to control. And there were some behavioural changes including posture, activity, roaming and arrogance were observed at higher dose treated group but no mortality was found throughout the experiment. On the other hand diet consumption was comparably same in all the treated groups compared to control group. The histophalogical changes observed in testis, epididymis and prostate gland may attribute to reduction of organ weight following NaCN treatment. These finding are in consistent with previous studies that shows cyanide cause poor body weight gain.[20,37]

Results from the current study showed that lower doses (0.64 mg/kg BW) of cyanide may not be a potential for the male reproductive toxicity in albino rats. This may be due to the existing detoxifying mechanism, which involves a mitochondrial enzyme rhodanase. Rhodanase can catalyze the reaction between CN^- and thiosulfate to produce SCN^-.[20] In which it transfer sulfur atom to CN^-.[38] And SCN^- is approximately 120 times less toxic than CN^-, and is excreted over several days.[39,40] In turn, cyanide can rapidly detoxify, and it enables animals to ingest sublethal doses of cyanide over extended periods without harm. However, the liver is highly sensitive to the cyanide and high doses of cyanide are beyond the detoxification capacity of the rhodanase system in the liver.[14,41]

The hormonal secretion from the pituitary, facilitate the normal spermatogenesis by paracrine and autocrine regulation of various components in the testis. Testosterone is the most important hormone involved in the regulation of the alterations observed in the testis of male rat, which is regulated by the pituitary-secreted gonadotropin hormones. Testosterone plays key role in the regulation of spermatogenesis, together with gonadotrophins. Its secretion from the Leydig cells is dependent on the secretion of LH from the pituitary gland.[42] In the present study the significant reduction in serum LH level of third ($P < 0.05$) and fourth ($P < 0.01$) group rats is a sign towards the possible effect of NaCN on the hypothalamus (Fig. 1B). This may be attributed to dysfunction of Leydig cells, which will lead to the decrease synthesis of testosterone in the testis (Fig. 1C). The FSH level was significantly declined in the fourth group treated with 3.2 mg/kg BW (Fig 1A). However, FSH was a very important hormone required for the proliferation of sertoli cells. And, the same was evidence by histopathological changes observed in the testis of the higher doses (1.2 and 3.2 mg/kg BW) treated rats (Fig. 2C,D). These findings were in consistent with the earlier reports.[43,44] Consequently, Sylvia *et al.,*[23] has demonstrated that subchronic cyanide treatment affects the spermatogenesis cycle through hypothalamic-pituitary-gonadal axis in the male rainbow trout.

In the present study, rats treated with 1.2 and 3.2 mg/kg BW, caused histopathological changes in testis, including atrophy and degenerated seminiferous tubules with cell debris in the lumina were observed (Fig. 2C,D). Ultimately, these changes have affected the spermatogonial cells, primary spermatid, secondary spermatid and spermatozoa. These histological changes in testis caused by NaCN led to low sperm counts, increased sperm abnormality (Table 2). Observed histopathological changes in the testis and alteration in sperm parameters might be attributed to oxidative stress and anaerobic cellular respiration.[45,46] Studies have demonstrated that cyanide induce oxidative stress in the functionally different tissues of rats.[47] Another possible cause for decreased sperm count, sperm motility and increased abnormal sperm in higher dose treated rats, was decreased availability of androgens (Fig. 1B,C). Sperms leaving the testis are not physiologically mature and such maturation takes place during their epididymal transit, after maturation, they become motile.[48] The changes in epididymis essentially involve the addition and modification of proteins by principal cells and removal of existing proteins by clearing cells.[49] These changes in the epididymal create

appropriate environment for spermatozoa to become mature.[50] It implies that if any histological changes in the epididymis may affect the maturation of the sperm. The notable histopathological changes include increased number of clearing cells and vacuolation in the epithelial cell layer of the epididymis at higher dose. (Fig. 3A,B). The sperm motility is a very important feature as it provides fertilizing capacity. Any negative impact on motility would seriously affect the fertilizing ability of sperms.[51] Studies demonstrate that cyanide can cause depletion in ATP synthesis in spermatozoa of crab by inhabiting the enzyme necessary for ATP synthesis.[52] Since, ATP plays crucial role in the forward movement of sperm.[53] Thus, reduced sperm motility on exposure to NaCN may be due to changes in the ATP pool. However, the secretion of the prostate is required to activate the sperms to fertilize the ovum and prostate in turn requires androgen for differentiation, development, and maintenance of epithelial cells.[54,55] Decreased prostate secretion in 1.2 and 3.2 mg/kg BW treated rats (Fig. 4C,D) may be due to the decreased serum testosterone level (Fig. 1C). Desquamation of tubuloalveolar glandular epithelial cells of prostate in the 3.2 mg/kg BW treated rats (Fig. 4D) could be attributed to either the decreased serum testosterone level or may be oxidative stress induced by NaCN in the current study.

From the results we conclude that subchronic exposure to low doses of cyanide may produce mild but the high dose (3.2 mg/kg BW) tested in the present study, induce adverse effect on male reproductive system in Wister strain albino rats. However, humans are more sensitive to the cyanide ions, sub-chronic exposure to the lower dose of cyanide might be leads to infertility in males. Therefore the care has to take while using cyanide in the industries.

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Table 1 Effect of sodium cyanide on body and reproductive organ weight.

Groups	Body Weight gain (g)	Organ weight		
		Testis (g)	Epididymis (g)	Prostate (g)
Control	96.42±6.1[a]	1.52±0.09a	0.39±0.2[a]	0.52±0.09[a]
0.64 mg/kg BW	86.42±5.08 [a]	1.44±0.03 [a]	0.35±0.1[a]	0.47±0.2[a]
1.2 mg/kg BW	86.53±2.6 [a]	1.30±1.2 [b]	0.33±0.1 [a]	0.44±0.2[b]
3.2 mg/kg BW	82.77±3.24 [b]	1.38±0.05[b]	0.29±0.2[b]	0.40±0.1[b]

The values (mean±SE) (n=7) bearing dissimilar letters in column differ significantly ($P < 0.05$).

Table 2 Sub-chronic effect of sodium cyanide on sperm motility, sperm morphology and abnormal sperm morphology in rats.

Groups	Parameters		
	Sperm count ($\times 10^6$/mL)	Sperm Motility (%)	Sperm Abnormality (%)
Control	292±3.7[a]	84.42±2.7[a]	8.67±0.46[a]
0.64 mg/kg BW	281±4.7[a]	80.71±3.83[a]	8.73±1.33[a]
1.2 mg/kg BW	264±8.6[b]	71.28±2.59[b]	10.23±2.91[a]
3.2 mg/kg BW	256±8.4 [b]	69.07±5.21 [b]	13.25±2.62[b]

The values (mean±SE) (n=7) bearing dissimilar letters in column differ significantly ($P < 0.05$).

401 **Table 3** Histopathological changes in the testis, epididymis and prostate of experimental rats, based
402 on scoring severity of injury in both organs.

Groups	Testicular injury		Epididymis injury	
	Score average (range)	Severity	Score average (range)	Severity
Control	-	Normal	-	Normal
0.64 mg/kg BW	-	Normal	-	Normal
1.2 mg/kg BW	+	Mild	+	Mild
3.2 mg/kg BW	++	Moderate	++	Moderate

403 Scoring was done as follows: none (-), mild (+), moderate (++) and severe (+++).

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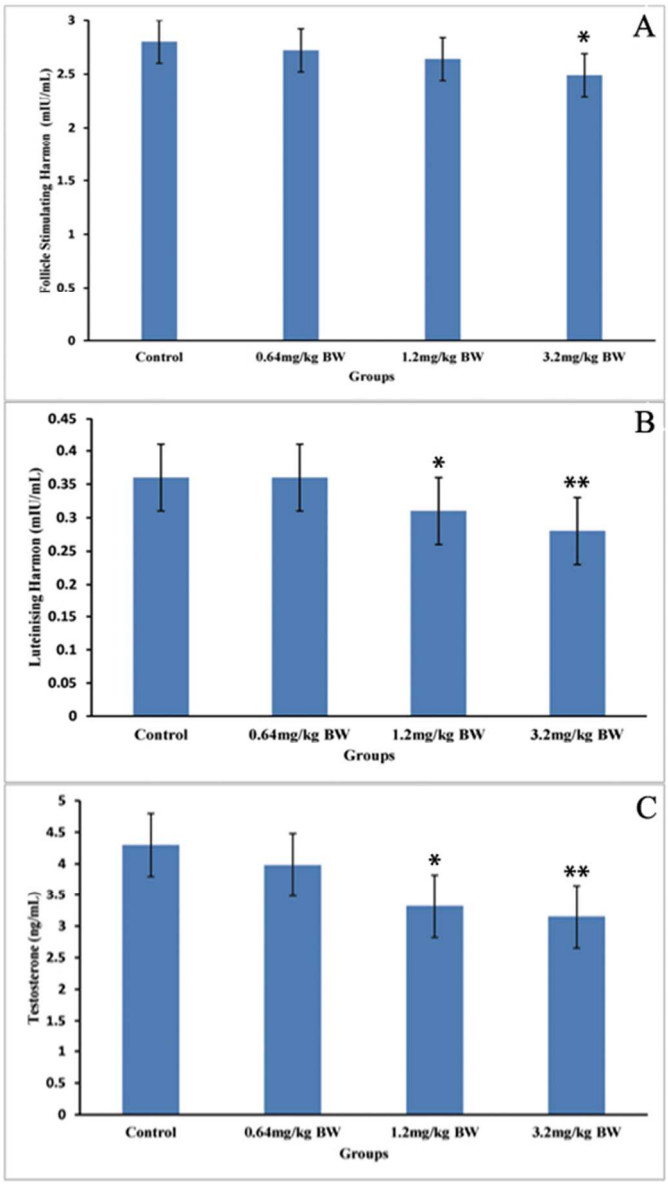

Fig. 1 Effect of NaCN on LH (a), FSH (b) and T (c) after subchronic exposure. The rats were exposed to different doses of sodium cyanide for 90 days. Bars represent mean±SEM. The asterisks above the bar denote significantly different from compared to control (*P < 0.05, **P < 0.01).
137x238mm (96 x 96 DPI)

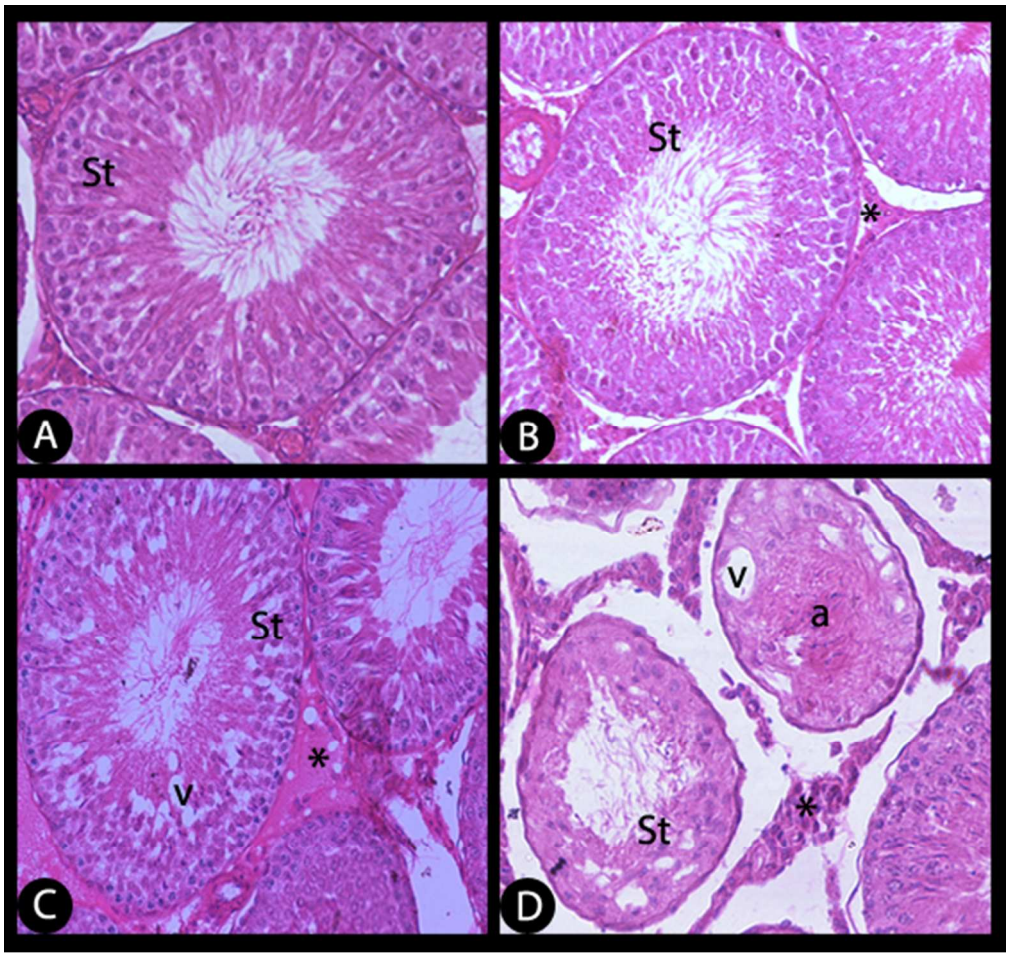

Fig. 2 H&E stained paraffin sections of testis; (A) Shows normal histoarchitecture of testis, seminiferous tubules (St), interstial tissue (*); (B) 0.64 mg/kg BW NaCN dosed testis sections shows normal histoarchitecture of testis, seminiferous tubules (St), intestinal tissue (*); (C) 1.2 mg/kg BW NaCN dosed testis sections shows vacuoles (v) in the germinal epithelial layers; (D) 3.2 mg/kg BW NaCN dosed testis sections shows vacuoles (v) in the germinal epithelial layers, atrophy (a) and degenerated seminiferous tubule (200x).
152x144mm (96 x 96 DPI)

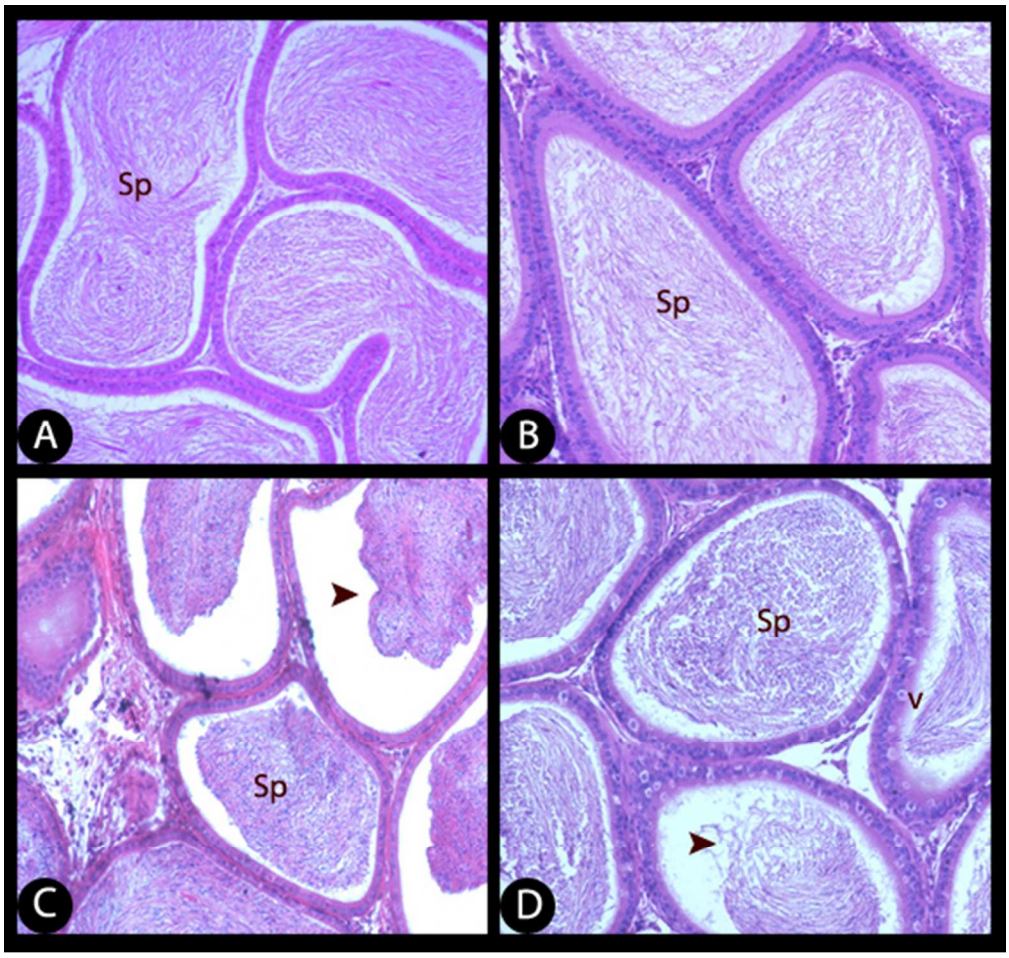

Fig. 3 H&E stained paraffin sections of epididymis; (A) Shows normal histoarchitecture of epididymis tubules with bulk of sperm (sp); (B) 0.64 mg/kg BW NaCN dosed epididymis shows normal histoarchitecture as seen in control group; (C) 1.2 mg/kg BW NaCN dosed epididymis low density of sperm; (D) 3.2 mg/kg BW NaCN dosed epididymis shows low sperm density and constricted at center, vacuoles (v) in the germinal epithelial cell lining (200x).
152x144mm (96 x 96 DPI)

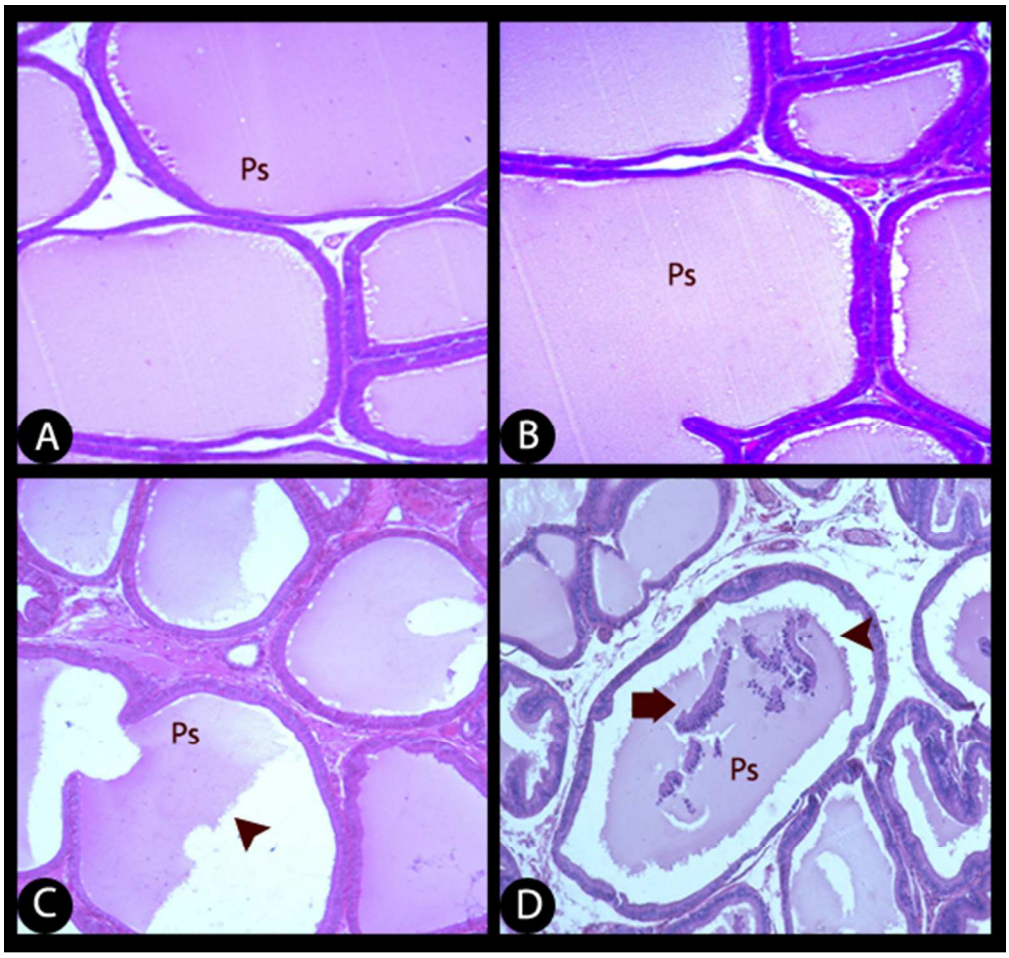

Fig. 4 H&E stained paraffin sections of prostate gland; (A) Shows normal histoarchitecture of prostate lumen with bulk of prostate secretion (Ps); (B) 0.64 mg/kg BW NaCN dosed rat prostate shows normal histoarchitecture; (C) 1.2 mg/kg BW NaCN dosed rat prostate shows low secretion (arrow head); (D) 3.2 mg/kg BW NaCN dosed rat prostate shows low secretion, desquamation of glandular epithelium (arrow) (200X).
152x144mm (96 x 96 DPI)