

The Reproductive Toxicity Assessment of Potassium Nitrate in Sprague Dawley Rats

F. W. Bansode¹ and R. K. Singh^{2*}

Divisions of Endocrinology, CSIR-Central Drug Research Institute, B.S. 10/1, Sector-10, Jankipuram Extension, Sitapur Road, Lucknow, U. P., India¹

Divisions of Toxicology, CSIR-Central Drug Research Institute, B.S. 10/1, Sector-10, Jankipuram Extension, Sitapur Road, Lucknow, U. P., India²

ABSTRACT: Epidemiologic evidence of a direct exposure-response relationship between drinking water nitrate level and adverse reproductive effects is still unclear. Present study was conducted to determine dose-dependent effects of potassium nitrate (KNO₃) on male reproductive function viz. Organ weights, spermatogenesis, daily sperm production rate (DSP), Leydig cell (LC) morphology, epididymal sperm counts (ESC) and steroidogenic enzymes activity, e.g. Δ^5 -3 β -Hydroxysteroid dehydrogenase (Δ^5 ,3 β -HSD) and 17 β -Hydroxysteroid dehydrogenase (17 β -HSD) in rats treated with 45, 90 and 135 mg/kg body weight for 15 days. Results showed no significant differences in water/food intake, body and reproductive organ weights in treated rats except significant decrease in prostate weight at 135mg/kg KNO₃ as compared to controls. Testis showed 40 \pm 8.9% abnormalcy exhibiting significant decrease in tubular/LC diameter, spermatocytes/spermatids number, DSP, ESC, Δ^5 -3 β -HSD and 17 β -HSD activity at 135mg/kg dose level than lower doses used. Results indicate testicular cytotoxicity by inhibition of spermatogenesis and steroidogenesis at 135mg/kg dose of KNO₃ in rats.

KEYWORDS: Potassium nitrate, Spermatogenesis, Steroidogenesis, Sperm counts, Rat.

I. INTRODUCTION

The haphazard use of nitrogenous fertilizers in agricultural activities and its contamination by human/animal organic waste on large-scale has led to increased concentration of nitrate in drinking water, food as well as in air [1, 2]. Nitrate, nitrite and ammonia are naturally occurring molecules in the environment and are the products of oxidation of nitrogen by microorganisms in plants tissues, soil and water [3, 4]. Nitrate is a salt of nitric acid, with an ion composed of one nitrogen and three oxygen atoms (NO₃⁻), and the nitrite (NO₂⁻, a salt of nitrous acid) can be formed in nitrate-containing and oxygen-poor water within galvanized steel pipes in the water distribution systems [2]. Ingestion of dietary (inorganic) nitrate elevates circulating and tissue levels of nitrite via bioconversion in the entero-salivary circulation and also causes dose-dependent elevation in plasma nitrite and cGMP concentration with a consequent decrease in blood pressure in healthy volunteers. Post hoc analysis demonstrated a sex difference in sensitivity to nitrate supplementation depending on resting baseline blood pressure and plasma nitrite concentration, whereby blood pressure is decreased in male volunteers with higher baseline blood pressure and lower plasma nitrite concentration but not in female volunteers [5, 6]. The dietary nitrate is reduced to nitrite which is converted to nitric oxide (NO) in acidic gastric juice and then NO in the stomach plays an important role in the protection of gastric mucosa from hazardous stress also [7]. Recent epidemiologic data have been suggested an association between developmental effects in offspring and the maternal ingestion of nitrate from drinking water, but a definite conclusion on the cause and effect relationship have not been yet drawn [4]. Previous studies have shown that the high levels of nitrate in water leads to increased the incidence of gastric cancer [8, 9], pulmonary lesions, blood disorders, retarded growth in children [10-13], hypertrophy of thyroid/thyroid disorders in rat and human [14-17], pregnancy-related disorders in rats, depression, muscle tremors in goats, sexual disorders in sheep [18], and reproductive/developmental effects [3, 19, 20, 21]. Rats

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

given 400 ppm sodium nitrite or sodium nitrate in drinking water for 6 months exerted immunosuppressive effect [22]. Nitrate has also shown mutagenic, embryotoxic and carcinogenic effects in experimental animals as well as in human [13, 23]. Several cases of methemoglobinemia have been reported in infants in the United States using water containing nitrate at levels higher than the current maximum contaminant level (MCL) of 45 ppm (mg/liter) NO_3 or 10 ppm nitrate-nitrogen, but none of the cases reported at lower doses than 45mg/L [10, 13, 24, 25-27]. The toxicity of nitrate to humans is due to endogenous reduction of nitrate to nitrite, which in turn may react with secondary amine to yield *N*-nitrosoamines, several of which are potent carcinogens in animals [28, 29]. Analysis of gastric contents after consumption of meals containing nitrite indicated that volatile nitrosamines may be formed under similar conditions *in vivo* [20]. Animal studies have shown some indication that nitrate, nitrite, and *N*-nitroso compounds may traverse the placenta and affect the foetus in utero [30, 31], and induce abortion in experimental animals [13, 24, 32], but showed no teratogenic effects. The fertility and gestation rates of sheep fed potassium nitrate were considerably lower (by 36% at 5 mg/kg body weight per day and 33% at 10 mg/kg body weight per day) and causes 32% decrease in number of oestrus sheep by 20mg/kg KNO_3 than in the control group [33] without affecting cyclic sexual behaviour. Existing animal experimental data have shown that reproductive toxicity is associated with high exposure levels to nitrate or nitrite, which are not likely to be encountered in drinking water. It includes spermatotoxicity in mice [34] and bulls [35] and inhibition of testosterone production and testicular size in male rat [36]. But, no consistent antiandrogenic effects were observed with nitrate exposure during pre-natal period and post natal development of dams [21]. The dose-dependent increase in weight and volume of the thyroid gland and decreased levels of triiodothyronine (T_3) and thyroxine (T_4) in rats and human exposed to nitrate in the drinking water have been reported at 150 and 500 mg/l of nitrate [15-17; 37]. The numerous endocrine disrupting chemicals, including certain anti-androgenic phthalates have been shown to decrease the anogenital distance (AGD) and impair steroidogenesis in foetal and neonatal male rats at critical periods of sexual development leading to malformations in the male reproductive system and reduced sperm count later in life [38-45]. However, information about nitrate toxicity is fragmentary and no definite conclusions can be drawn due to lack of pertinent male reproductive toxicity data in the literature. The present study was conducted to determine the dose-dependent effects of KNO_3 to which the human population is exposed to nitrate in the drinking water in certain parts of India as well as at its higher dose level, on the male reproductive organs function viz. organs weights, quantitative analysis of testicular spermatogenesis, DSP, epididymal sperm count, key testicular androgenic enzymes activity eg. Δ^5 , 3β -hydroxysteroid dehydrogenase (Δ^5 , 3β -HSD) and 17β -hydroxysteroid Dehydrogenase (17β -HSD) and Leydig cell morphology in adult Sprague Dawley rats.

II. MATERIALS AND METHODS

Chemicals: Chemicals including haematoxylin, eosin, NaCl, NaN_3 , Triton X-100, KCl, potassium phosphate, sodium pyrophosphate, Na_2HPO_4 , KH_2PO_4 , D-glucose, testosterone, Dehydroepiandrosterone (DHEA), EDTA, BSA, NAD, NAD^+ and Bradford reagent were purchased from Sigma Chemicals Company, USA. Potassium nitrate (98.5% pure) was procured from Merck Limited, Mumbai, India. All other chemicals of analytical grade were purchased locally.

Animals, treatment and tissue fixation: Adult male Sprague Dawley rats (180-200g Body weight) obtained from the Institute's breeding colony were acclimatized to uniform husbandry conditions ($22 \pm 3^\circ\text{C}$, 12 h light: 12 h dark cycle) for 1 week prior to the experiment. Animals were fed with measured amount of food (a pellet diet supplied by Hindustan Lever Ltd., Bangalore) and water daily to access food and water intake. All rats were handled in accordance with the standard guide for the care and use of laboratory animals, and experimental protocol was approved by Institutional Animal Ethics Committee (IAEC).

48 adult male rats were randomly divided into four groups (Gr. I-IV) containing 12 animals in each group. Rats in Gr. I were given vehicle (10ml/kg distilled water) only and served as control. Animals in Gr. II, III and IV were administered orally with 45, 90 and 135 mg/kg body weight of potassium nitrate (dissolved in distilled water), respectively, for 15 days. Food intake, water intake, and gross behavioural changes were recorded daily for 1-2 hr after the drug

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

administration. Autopsy of animals from control and treated groups was done on day 16 after 15 days of treatment. The reproductive organs viz. testes, epididymis and accessory sex organs (ventral prostate and seminal vesicles) were dissected out, rinsed immediately in chilled saline, freed from connective tissues and blood clots, weighed and left side testes and epididymis fixed in Bouin's fluid (24 h) for histology purpose. Tissues from right side were used for quantitation of daily sperm production rate in testis, biochemical estimation of enzymes (Δ^5 -3 β HSD and 17 β -HSD) and epididymal sperm counts, etc.

Histo-morphometry of testis and epididymis: Bouin's fixed testes and epididymis from control and treated groups of rats were dehydrated in graded series (50-100%) of ethanol, cleared in xylene, and infiltrated and embedded in paraffin wax (at 58°C). Serial cross sections (5 μ m) were stained with haematoxylin and eosin. Measurements of testicular tubular diameter (20 tubules each rat with total of 100 tubules/ group) and the nuclear diameter of Leydig cells (20 Leydig cells each rat with total of 100 cells/group) were carried out under Olympus Trinocular Microscope (Olympus BX 51, Japan) at x400 and x1000 (oil immersion) magnification respectively using Biovis Image Plus Software for image analysis and processing (Expert Vision Labs Pvt. Ltd. Mumbai, India).

Quantitative analysis of spermatogenesis: Quantitative analysis of spermatogenesis was carried out as per method of Srivastava et al. [46] based on nuclear morphology of germ cells and Sertoli cells. The number of germ and Sertoli cell nuclei were counted in at least 20 round seminiferous tubules selected randomly from at least 2-3 cross sections in each rat. The number of germ cells viz. A-spermatogonia, non-pachytene (pre-leptotene, leptotene and zygotene) and pachytene spermatocytes, round and elongated spermatids were assessed in each group of 5 rats at 400X magnification under Olympus Trinocular Microscope (Olympus, BX 51, Japan). The cell counts for different germ cell types were corrected by Abercrombie's formula [47].

Estimation of daily sperm production (DSP): Measurement of DSP in testicular homogenates was carried out as per method of Amann et al. [48]. Briefly, testicular parenchyma was cut into small pieces, placed in 0.25 mol/l sucrose solution (pH 7.5) and homogenized in a fluid containing 150 mM NaCl/l, 3.8 mM NaN₃/l, and 0.05% (v/v) Triton X-100 using an Ultra Turrax® (Janke and Kunkel, Staufen, Germany). The homogenates were further diluted with the same medium, and homogenization-resistant spermatid nuclei in steps 17-19 of stages IV-VIII of the spermatogenic cycle were counted in triplicate under the Olympus microscope using a Neubauer's haemocytometer [49]. DSP per gram testicular parenchyma was calculated by dividing the number of spermatid nuclei with the product of weight of parenchyma and time divisor of 6.3 days reported for rat [49]. The values are represented as mean \pm se for six animals in each group.

Epididymal sperm count: Cauda epididymis was excised surgically, cut into small pieces in suspension medium containing 140 mmol NaCl, 0.3 mmol KCl, 0.8 mmol Na₂HPO₄, 0.2 mmol KH₂PO₄ and 1.5 mmol D-glucose (pH adjusted to 7.3 by adding 0.1 (N) NaOH) and sperms were collected by centrifugation at 100 \times g for 2 min. The resultant precipitate was resuspended in fresh suspension medium, diluted and placed on both sides of Neubauer's hemocytometer. The counting of number of sperms was carried out in four chambers of hemocytometer in duplicate in each rat under Olympus Trinocular microscope at x100 magnification. The values were expressed as an average of total counts of sperm number per ml of suspension [46, 50].

Estimation of Δ^5 , 3 β - and 17 β - Hydroxysteroid dehydrogenase activity: The testicular Δ^5 , 3 β -Hydroxysteroid dehydrogenase (Δ^5 , 3 β -HSD) and 17 β - Hydroxysteroid dehydrogenase (17 β -HSD) enzyme activity was measured according to the methods described previously [51, 52]. The testis was encapsulated and tissues of each animal was homogenized in 15% glycerol containing 5 mmol potassium phosphate and 1 mmol EDTA at a tissue concentration of 100 mg/ml. The homogenates were centrifuged at 10,000 \times g for 30 min at 4°C. For Δ^5 , 3 β -HSD enzyme activity, the supernatant (1 ml) was mixed with 1 ml of 100 μ mol sodium pyrophosphate buffer (pH 8.9) and 30 μ g of dehydroepiandrosterone (Sigma, USA) in 40 μ l of ethanol and 960 μ l of 25% BSA (Sigma, USA), making the incubation mixture to 3 ml. The enzyme activity was measured after addition of 0.5 μ mol of NAD (Sigma, USA) to the

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

tissue supernatant mixture using a U2000 spectrophotometer (Hitachi, Japan) cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity is the amount causing a change in absorbance of 0.001/min at 340 nm. The activity of testicular 17 β -HSD was measured using the same supernatant prepared for the assay of Δ^5 , 3 β -HSD (above). The supernatant (1 ml) was mixed with 1 ml of 440 μ mol sodium pyrophosphate buffer (pH 10.2), 40 μ l of ethanol containing 0.3 μ mol of testosterone (Sigma, USA) and 960 μ l of 25% BSA (Sigma), making the incubation mixture a total of 3 ml. The enzyme activity was measured after addition of 1.1 μ mol NAD (Sigma) to the tissue supernatant mixture in a U2000 spectrophotometer cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

Protein estimation: Bradford Assay [53] was used to determine the total protein concentration of the samples from control and treated rats in thrice. Different dilutions (1 μ g to 10 μ g) of 1% BSA in different eppendorfs were made. 5 μ l from each sample using eppendorf was taken in different wells of ELIZA plates. Then added 150 μ l of Bradford reagent (Sigma Chem. Co., USA) to each well. Covered the plate with aluminium foil as Bradford reagent is light sensitive and read the O.D. at 595 nm with the help of μ Quant ELISA reader. Plotted a Standard graph of protein estimation with different dilutions of BSA concentration and O.D. obtained. Testicular extracts (100 mg/ml) were prepared in homogenizing fluid, ultra-centrifuged at x10000 rpm for 30 minutes. Supernatant was separated. In ELIZA plate (96 well plates) 190 μ l Bradford reagent+5 μ l samples was added and read at 595 nm wavelength. O.D. was recorded and Concentrations of protein in testicular tissue was calculated using Standard BSA plot. Three readings per sample were taken.

Statistical analysis: Statistical analysis for significant differences between control and treated group of animals was done by Students 't' test and one-way ANOVA (one factor analysis of variance). Values with P < 0.05 were considered as significant. Values were expressed as Mean \pm SE.

III. RESULTS

Body and reproductive organ weights: Body weights did not show any significant change in rats treated with KNO₃ at the doses of 45 mg/kg (Gr. II), 90 mg/kg (Gr. III) and 135 mg/kg (Gr. IV) for 15 days as compared to control (Gr. I) rats. The reproductive organ weights viz. testes, epididymis, prostate and seminal vesicles did not show any significant change in treated as compared to in control rats (Table 1).

Table 1. Effect of potassium nitrate on body and reproductive organ weights in male rats after 15 days of treatment.

Groups/Treatments	Body weights	Reproductive organ weight (g)			
		Testis	Epididymis	Ventral Prostate	Seminal vesicles
I Control	241.5 \pm 6.5	1.553 \pm 0.045	0.640 \pm 0.039	0.437 \pm 0.037	1.055 \pm 0.134
II. KNO ₃ (45 mg/kg)	241.0 \pm 6.7 ^{NS}	1.556 \pm 0.095 ^{NS}	0.679 \pm 0.032 ^{NS}	0.306 \pm 0.04 ^{NS}	1.280 \pm 0.089 ^{NS}
III. KNO ₃ (90 mg/kg)	235.9 \pm 5.9 ^{NS}	1.374 \pm 0.139 ^{NS}	0.628 \pm 0.071	0.337 \pm 0.013	1.053 \pm 0.143 ^{NS}
IV. KNO ₃ (135 mg/kg)	223.6 \pm 2.2	0.894 \pm 0.062	0.543 \pm 0.02	0.331 \pm 0.02 ^a	0.823 \pm 0.033

Values expressed as mean \pm s.e.m., n=10 number of animals, Significance between treated -vs.- control, NS – Non-significant, a – p<0.02

Histo-morphometry of testis: In control rats, testicular seminiferous epithelium showed all germ cell types, eg. Spermatogonia, Non-pachytene (preleptotene, leptotene, zygotene) and pachytene primary spermatocytes, spermatids (round and elongated) and spermiating spermatozoa. The Sertoli and Leydig cells showed normal morphology. In rats treated with 45 and 90 mg/kg, all germ cells types and Sertoli cells in the seminiferous epithelium, and interstitial Leydig cells showed normalcy. In contrast, rats treated with 135 mg/kg dose of KNO₃ caused seminiferous epithelial

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

destruction in $40 \pm 8.9\%$ testicular tubules (Abnormal tubules). The degenerative changes in abnormal tubules were marked by cytoplasmic vacuolization, multinuclear giant cell formation, desquamation and degeneration of germ cells into tubule lumen and disintegration of spermatozoa. Some of the testicular tubules exhibited complete destruction of germ cells up to primary spermatocytes stage and presence of only the spermatogoniae and Sertoli cells was observed. However, $60 \pm 8.9\%$ testicular tubules showed normal seminiferous epithelium exhibiting all germ cell types at 135mg/kg dose. The seminiferous tubular diameter and diameter of Leydig cells nuclei showed significant reduction ($p < 0.001$) in abnormal tubules as compared to normal tubules/controls. The Leydig cells between affected tubules displayed hyperplasia with increased leucocytes infiltration at 135 mg/kg dose of KNO_3 . (Table 2, Fig. 1 A-F).

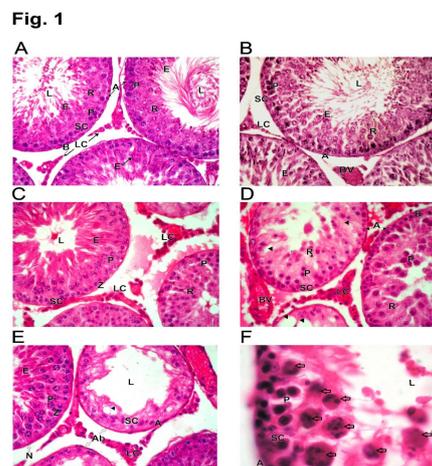


Figure 1. Testis histology of control (A) and potassium nitrate (KNO_3)-treated (B-F) rats. Note the normal testicular structure showing all germ cell types viz. spermatogonia, spermatocytes, spermatids and spermatozoa in seminiferous epithelium of control (A) rats. In rats treated with 45 mg/kg (B) of KNO_3 show normal testicular structure. Sertoli and Leydig cells show normalcy. In treated rats at 90 mg/kg (C) KMNO_3 showed degeneration and disintegration of germ cells in testicular tubules. (D-F) Testis of 135 mg/kg KNO_3 -treated rat showing degeneration of pachytene spermatocytes, round spermatids and absence of elongated spermatids/luminal spermatozoa in seminiferous tubules. Cytoplasmic vacuolization (arrow head) can be seen in the seminiferous epithelium. Leydig cells show attenuated profile. (E) Figure showing normal (N) testicular tubule depicting all germ cell types, while abnormal (Ab) tubule exhibiting absence of germ cells except spermatogonia and Sertoli cells. (F) Testicular cross section showing multinucleated giant cell formation (arrow) and disintegrated spermatozoa in 135mg/kg KNO_3 treated rat. A,B- spermatogonia, Z- Zygote spermatocytes, P- Pachytene spermatocytes, R- Round spermatids, E- Elongated spermatids/spermatozoa, L- Lumen, SC- Sertoli cells, LC- Leydig cells, N-Normal tubule, Ab- Abnormal tubules. Figures A-E, x400 magnification and F, x1000 (under oil immersion); Haematoxylin-Eosin.

Quantitation of spermatogenesis: Quantitative analysis of spermatogenesis carried out in rats treated with 45 and 90 mg/kg of KNO_3 did not show any significant difference in the number of all germ cell types and Sertoli cell number when compared with control rats. However, in rats treated with 135mg/kg dose of KNO_3 , showed significant decrease in number of primary spermatocytes (non-pachytene and pachytene) and spermatids (round and elongated) in normal as well as in abnormal tubules as compared to controls (Table 2, Fig. 2).

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

Table 2. Quantitative analysis of testicular spermatogenesis, tubular diameter, nuclear diameter of Leydig cells and % age of abnormal (affected tubules showing incomplete stages of spermatogenesis) tubules in control and potassium nitrate (KNO₃) treated rats for 15 days.

Groups/ diameter Treatments	Germ cells / Round seminiferous tubule					Sertoli cells /tubule	% age of Abnormal Tubules	Tubular diameter (μ m)	Nuclear of Leydig cells (μ m)
	A	N-P	P	RS	ES				
I. Control	01.64 \pm 0.1	15.0 \pm 0.6	14.9 \pm 0.8	51.2 \pm 2.3	68.6 \pm 3.2	12.7 \pm 0.3	-	511.4 \pm 11.6	13.6 \pm 1.5
II. (45mg) KNO ₃ -Treated	1.71 \pm 0.1 ^{NS}	15.2 \pm 0.7 ^{NS}	14.9 \pm 0.8 ^{NS}	47.14 \pm 1.7 ^{NS}	64.6 \pm 2.5 ^{NS}	12.4 \pm 0.2 ^{NS}	-	495.2 \pm 8.3 ^{NS}	12.4 \pm 1.2 ^{NS}
III. (90mg)	1.81 \pm 0.1 ^{NS}	15.9 \pm 0.6 ^{NS}	14.0 \pm 0.7 ^{NS}	46.0 \pm 2.9 ^{NS}	61.8 \pm 2.2 ^{NS}	12.2 \pm 0.2 ^{NS}	-	509.9 \pm 11.2 ^{NS}	13.2 \pm 1.1 ^{NS}
IV. (135mg)N	1.59 \pm 0.1 ^{NS}	13.3 \pm 0.6 ^a	11.5 \pm 0.7 ^c	34.2 \pm 1.8 ^b	44.5 \pm 2.1 ^d	13.0 \pm 0.4 ^{NS}	-	477.4 \pm 14.3 ^a	11.3 \pm 0.5 ^{NS}
IV. (135mg)Ab	1.69 \pm 0.2 ^{NS}	10.6 \pm 0.9 ^d	7.7 \pm 0.68 ^d	9.2 \pm 1.7 ^d	8.65 \pm 2.9 ^d	12.5 \pm 0.5 ^{NS}	40.0 \pm 8.9	296.3 \pm 9.1 ^d	08.5 \pm 0.3 ^a

Values expressed as mean \pm s.e.m., n=6 number of animals, A- Spermatogonia, N-P – Non-pachytene spermatocytes, P- Pachytene spermatocytes, RS- Round spermatids, ES- Elongated spermatids, N- Normal tubules (displaying all germ cell types), Ab- Abnormal tubules (tubules with incomplete stages of spermatogenesis); Significance between control and treated groups, NS - Not significant, a – p<0.05, b – p<0.01, c – p<0.005, d – p<0.001.

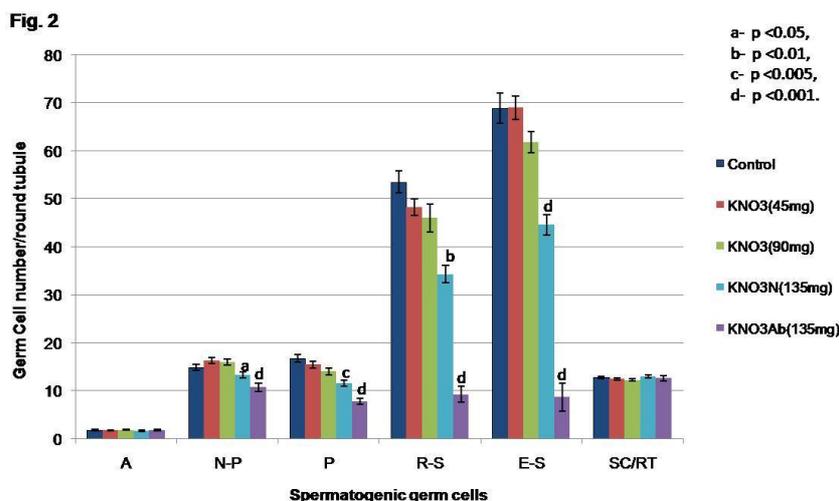


Figure 2. Histogram showing quantitative analysis of the germ cells number /round tubule in control and KNO₃-treated rats with 45 mg/kg, 90 mg/kg and 135 mg/kg doses. A-Spermatogonia, NP-Non-pachytene spermatocytes, P-Pachytene spermatocytes, RS- Round spermatids, ES- Elongated spermatids, SC/RT- Sertoli cells/round tubule, N-Normal tubules, Ab- Abnormal tubules.

Estimation of daily sperm production (DSP): Estimation of daily sperm production rate in testicular homogenates of rats treated with 45 or 90 mg/kg of KNO₃ for 15 days showed no significant change as compared to controls. However, a significant decrease (p<0.02) in DSP rate was observed in rats treated with higher dose (135 mg/kg/day) of KNO₃ as compared to control rats (Fig. 3 A).

Epididymal sperm counts: Cauda epididymal sperm counts in rats treated with 45 and 90 mg/kg KNO₃ did not show any significant decrease but, exhibited a significant decrease (p<0.05) in number of sperms at 135 mg/kg dose as compared to controls (Fig. 3 B).

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

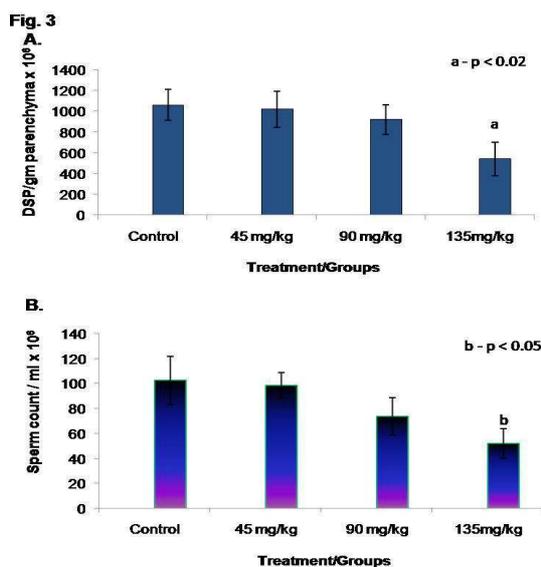


Figure 3. Testicular daily sperm production (DSP/gm parenchyma x 10⁶) (in Fig. A) and cauda epididymal sperm counts/ml x 10⁶ (in Fig. B) in control and KNO₃-treated rats.

Effects of potassium nitrate on enzymatic analysis: There was a significant decrease in testicular estimation of steroidogenic enzymes, viz. Δ^5 -3 β -HSD (Fig. 4 A) and 17 β -HSD (Fig. 4 B) activity in rats treated with 135mg/kg dose of KNO₃ for 15 days as compared to control rats. Whereas, in rats treated with lower doses (45 and 90 mg/kg) of KNO₃ did not show any significant change in both the enzymatic activity.

Fig. 4

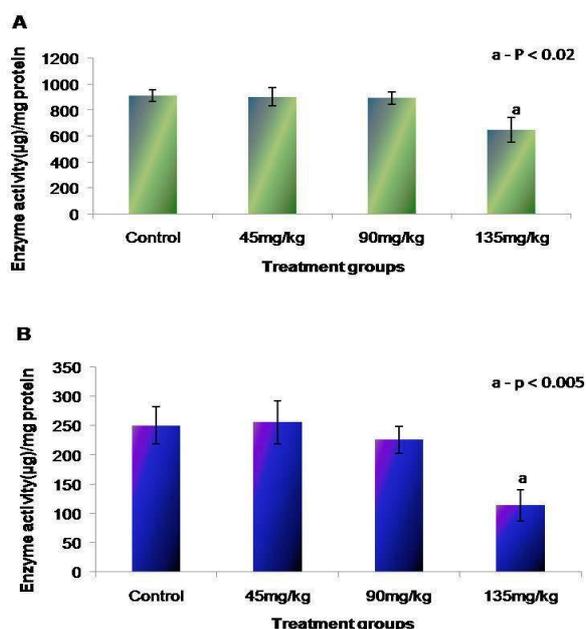


Figure 4. Figure showing testicular Δ^5 3 β -Hydroxystroid dehydrogenase enzyme activity (A) and 17 β -Hydroxystroid dehydrogenase enzyme activity (B) in control and KNO₃-treated rats.

Food and water Intake: There was no difference in the uptake of water and food consumption in control and treated animals during the entire treatment period (Fig. 5 A, B).

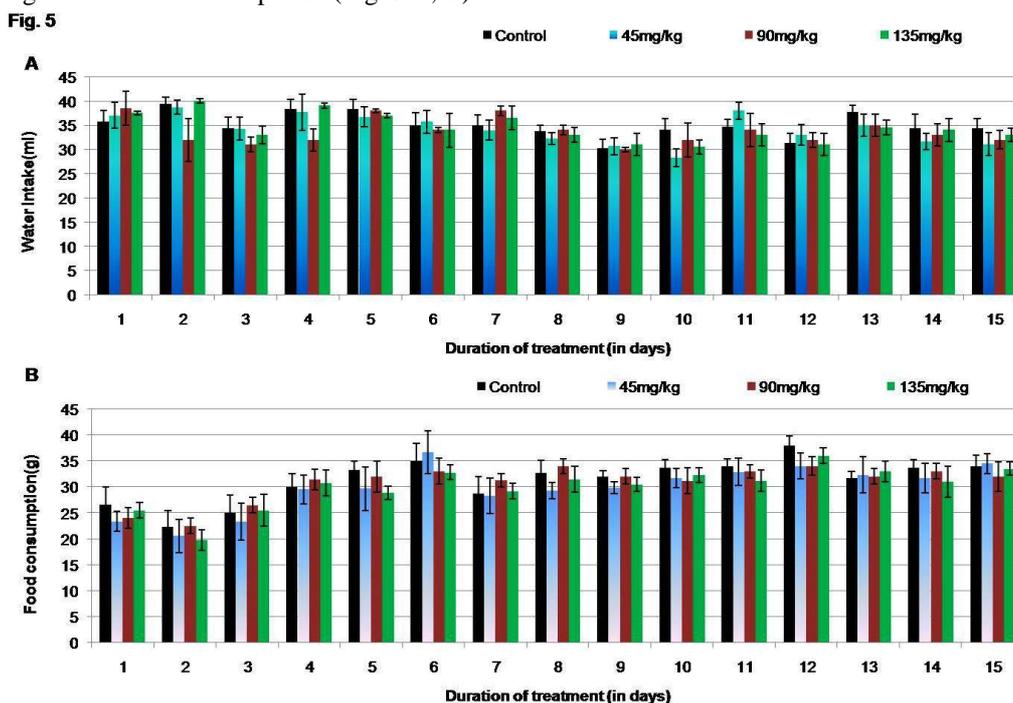


Figure 5. Intake of water (A) and food consumption (B) in control and KNO₃-treated rats during 15 days of treatment period.

Toxicity study: The animals remained apparently normal during entire treatment period, except for development of dullness in 135 mg/kg KNO₃ treated animals.

IV. DISCUSSION

Present study was conducted to determine dose-dependent effects of KNO₃ on male reproductive organs with special emphasis on testicular toxicity. Results of the present study did not show any significant changes in the body weights and reproductive organ weights in rats treated with KNO₃ at 45, 90 and 135 mg/kg doses for 15 days, except for a significant decrease ($p < 0.02$) in weight of ventral prostate gland at 135 mg/kg dose. Previous study with KNO₃ in mice has also been shown no any significant difference in body and reproductive organ weights at higher dose of 900 ppm in mice [34, 54]. The morphometric analysis of testes carried out in the present study showed $40 \pm 8.9\%$ abnormal testicular tubules (with incomplete stages of spermatogenesis) and $60 \pm 8.9\%$ Normal tubules (with complete spermatogenesis depicting all germ cell types) only at 135 mg/kg dose of KNO₃. The quantitative analysis of spermatogenesis in treated rats showed a significant inhibition of primary spermatocytes viz. non-pachytene ($p < 0.05-0.001$) and pachytene ($p < 0.005-0.001$), and spermatids (round and elongated in steps 1-19 of spermiogenesis, $p < 0.001$) in both normal and abnormal tubules only at 135 mg/kg dose level as compared to controls (Table 2). In contrast, the number of spermatogonia-A and Sertoli cells were observed to be unchanged than in control rats. The inhibition of germ cells in higher dose (135 mg/kg) of KNO₃ was also associated with decreased daily sperm production rate in testicular homogenates as well as cauda epididymal sperm counts in treated rats. The suppression of spermatogenic germ cells was also accompanied by extensive cytotoxicity in seminiferous epithelium depicting cytoplasmic vacuolization, giant cell formation, pyknosis of nuclei, desquamation and dissolution of germ cells in tubule lumen and disintegration of spermatozoa in abnormal tubules of treated (135 mg/kg) rats. Further, KNO₃ treatment also caused

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

significant decrease ($p < 0.001$) in testicular tubular diameters (in both normal and abnormal tubules) at 135 mg/kg dose level as compared to controls. However, in rats treated with 45 and 90 mg/kg doses of KNO_3 , indicated no significant changes as compared to in controls. Our results, are in agreement with the previously reported studies that indicate no any detectable histopathological change at lower doses (90, 200, 500 and 700 ppm) of KNO_3 in mice, but revealed moderate atrophy with disturbed spermatogenesis, prominent interstitial space at 900-ppm congestion (Pant and Srivastava, 2002) and spermatotoxicity in rat [29] and bulls [35]. Results of the study also showed significant inhibition of androgenic function evidenced by reduced size and morphology of Leydig cells and significant decline ($p < 0.02$) in accessory sex organ (prostate) weight at 135 mg/kg KNO_3 treatment for 15 days as compared to controls. Existing reported studies have been shown the inhibition of testosterone production and testicular size in nitrate and nitrite exposure via drinking water [36]. It has been also reported that KNO_3 treatment causes an increase in weight of thyroid gland but, decreased thyroxin hormone levels [15, 16]. In contrast, lower doses of sodium nitrate did not inhibit testosterone level in wister rats [29]. In pregnant dams, nitrate and nitrite exposure from days 7 to 21 of gestation at 17.5, 50, 450 and 900 mg/l did not affect the testosterone, progesterone and thyroxin levels and shown no histopathological changes during sexual development of male foetuses [21]. Further, studies on testosterone deprivation have been shown to suppress plasma LH, nuclear morphology of Leydig cells and germ cells, while testosterone supplementation restores normal function of Leydig cells and spermatogenesis indicating testosterone dependent cellular sensitivity of Leydig cells [55-58]. The anti-androgenic phthalates exposure to prenatal period have been also demonstrated to show impairment of steroidogenesis in foetal and neonatal male rats leading to malformation in male reproductive system and reduction in the sperm count in later life [38-40]. Results of the present study on biochemical estimation of key steroidogenic enzymes, Δ^5 3 β -HSD and 17 β -HSD activity [59-61] in testicular tissue, indicated significant decrease in KNO_3 -treated rats at 135 mg/kg dose for 15 days. Similar decrease in testicular 17 β -HSD activity along with an increase in glutamyl transpeptidase activity in KNO_3 -treated mice has been reported at higher dose level previously [34, 62, 63]. The inhibition of Δ^5 , 3 β -HSD and 17 β -HSD activity in testis may indicate inhibition of steroidogenesis in KNO_3 -treated rats at 135 mg/kg dose level only. It was also accompanied with decreased nuclear diameter of Leydig cells, androgen-dependant primary spermatocytes (nonpachytene and pachytene) and spermatids (round and elongated) as well as deduction in weight of prostate gland at higher dose level rather than its lower doses. The inhibition of steroidogenesis and spermatogenesis may be due to the direct effect of nitrate or its metabolites or due to low levels of plasma gonadotrophins (plasma FSH and LH) responsible for regulating its activity in testis. It has been demonstrated that the nitrate interferes with the normal physiology of Sertoli cells which may affect the development of androgen-dependant germ cells (viz. spermatocytes, spermatids) and increases sperm abnormality as well as declines sperm counts [34] by 900ppm KNO_3 treatment for 35 days in mice [34], rats [29] and Bulls [35]. This loss of sperm function has been reported to be associated with excessive free radical generation in sperm and seminal plasma [64]. Our results at 135 mg/kg dose of KNO_3 have been also shown a significant decrease in testicular daily sperm production, germ cell population and epididymal sperm counts. It indicate that exposure to high concentration of nitrate could adversely effect the development of germ cells and as evidenced by decline spermatogenic gem cells, steroidogenic enzyme activity, Leydig cell size and histopathology may indicate testosterone inhibition [65]. Reported studies with other compounds like carbaryl, carbofuran, arsenic chromium, lead, benzene, chloroform, phenol and trichloroethylene [66], other toxicants like lupeol acetate [67], intragastric perfusion of waste [68], benzene exposure [68-70] as well as other pollutants/ chemical agents as well as estrogens/antiestrogens/androgens have been shown to cause depletion of primary spermatocytes, spermatids and spermatotoxicity including inhibition of semen quality, sperm counts, sperm motility, total spermatozoa/ejaculates, daily sperm production, acrosomal dysgenesis, nuclear malformations, mating desire/ability, sperm quality and Leydig cell function associated with decreased serum concentrations of LH and Testosterone secretion and the activity of marker testicular enzymes associated with specific cell types and histopathological parameters [54, 55, 71-78]. Though the exact mechanism of action of KNO_3 treatment is still not clear, but, it may cause a reproductive toxicity only at higher dose level, which is not likely to be encountered in drinking water in human. Further, chronic administration of KNO_3 is needed to examine the adverse effects if any and to explore its exact mechanism of action more precisely by keeping in view the continuous exposure to human beings in actual prevailing condition.

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

V. CONCLUSION

The present study demonstrates the antispermatogenic effects of KNO_3 at 135mg/kg dose level for 15 days treatment in rats than that of its lower doses (45 and 90 mg/kg), which did not show any effect of testicular spermatogenesis. Inhibition of testicular spermatogenesis was evident by depletion of androgen-dependent meiotic primary spermatocytes (non-pachytene and pachytene) and post-meiotic spermatids (round and elongated), inhibition of testicular tubular diameter, DSP, key steroidogenic enzymes (Δ^5 3 β -HSD and 17 β -HSD) activity, nuclear diameter of Leydig cells, epididymal sperm count and accessory sex organ (ventral prostate) weight at 135 mg/kg dose level, may indicate antispermatogenic and anti-androgenic effects of KNO_3 at 135 mg/kg dose level in rat.

ACKNOWLEDGEMENTS

The authors are thankful to Ms. T. Firdaus and Mr. Nand Pal Yadav from Endocrinology and Toxicology Division for technical assistance. This work was funded by Ministry of Health and Family Welfare, New Delhi, India.

REFERENCES

- [1] Romano, N., Zeng, C., "Acute toxicity of sodium nitrate, potassium nitrate, and potassium chloride and their effects on the hemolymph composition and gill structure of early juvenile blue swimmer crabs (*Portunus pelagicus* Linnaeus, 1758) (decapoda, brachyura, portunidae)", Environ. Toxicol. Chem., Vol. 26, pp. 1955-1962, 2007.
- [2] World Health Organization, "Nitrate and nitrite in drinking water: Background document for development of WHO Guidelines for Drinking-water Quality", 2007.
- [3] Manassaram, D.M., Backer, L.C., Moll, D.M., "A review of nitrates in drinking water: Maternal exposure and adverse reproductive and developmental outcomes", Environ. Health Perspect., Vol. 114, pp. 320-327, 2006.
- [4] Manassaram, D.M., Backer, L.C., Moll, D.M., "A review of nitrates in drinking water: maternal exposure and adverse reproductive and developmental outcomes", Cien Saude Colet., Vol. 12, pp. 153-163, 2007.
- [5] Kapil, V., Milsom, A.B., Okorie, M., Maleki-Toyserkani, S., Akram, F., Rehman, F., Arghandawi, S., Pearl, V., Benjamin, N., Loukogeorgakis, S., Macallister, R., Hobbs, A.J., Webb, A.J., Ahluwalia, A., "Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO", Hypertension, Vol. 56, pp. 274-281, 2010.
- [6] Vanhatalo, A., Bailey, S.J., Blackwell, J.R., DiMenna, F.J., Pavey, T.G., Wilkerson, D.P., Benjamin, N., Winyard, P.G., Jones, A.M., "Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise", Am J Physiol Regul Integr Comp Physiol., Vol. 299, pp. R1121-1131, 2010.
- [7] Miyoshi, M., Kasahara, E., Park, A.M., Hiramoto, K., Minamiyama, Y., Takemura, S., Sato, E.F., Inoue, M., "Dietary nitrate inhibits stress-induced gastric mucosal injury in the rat", Free Radic. Res., Vol. 37, pp. 85-90, 2003.
- [8] Dull, B. J., Hotchkiss, J. H., "A rapid method for estimating nitrate in biological samples using gas chromatography with a flame ionization detector or a thermal analyser", Food Chem. Toxicol., Vol. 22, pp. 105-108, 1984.
- [9] Gulis, G., Czompolyva, M., Cerham, J.R., "An ecologic study of nitrate in municipal drinking water and cancer incidence in Trnava District, Slovakia", Environ. Res. Sec. A., Vol. 88, pp. 182-187, 2002.
- [10] Chow, C.K., Chen, C.J., Gairola, C., "Effect of nitrate and nitrite in drinking water on rats", Toxicol. Lett., Vol. 6, pp. 199-206, 1980.
- [12] Johnson, C.J., Kross, B.C., "Continuing importance of nitrate contamination of ground water and wells in rural areas", Am. J. Ind. Med., Vol. 13, pp. 449-456, 1991.
- [13] Fan, A.M., Steinberg, V.E., "Health Implications of Nitrate and Nitrite in Drinking Water: An Update on Methemoglobinemia Occurrence and Reproductive and Developmental Toxicity", Regul. Toxicol. Pharmacol., Vol. 23, pp. 35-43, 1996.
- [14] Johnson, L., Nguyen-Hung, B.A., Petty, C.S., Neaves, W.B., "Quantification of human spermatogenesis. Germ cell degeneration during spermatogenesis and meiosis in testes from younger and older adult men", Biol. Reprod., Vol. 37, pp. 739-747, 1987.
- [15] Zaki, A., Ait Chaoui, A., Talibi, A., Derouiche, A.F., Aboussaouira, T., Zarrouck, K., Chait, A., Himmi, T., "Impact of nitrate intake in drinking water on the thyroid gland activity in male rat", Toxicol. Lett., Vol. 147, pp. 27-33, 2004.
- [16] Esciocak, S., Dundar, C., Basoglu, T., Altaner, S., "The effects of taking chronic nitrate by drinking water on thyroid functions and morphology", Clin. Exp. Med., Vol. 5, pp. 66-71, 2005.
- [17] Tajtakova, M., Semanova, Z., Tomkova, Z., Szokeova, E., Majoros, J., Radikova, Z., Sebkova, E., Klimes, I., Langer, P., "Increased thyroid volume and frequency of thyroid disorders signs in schoolchildren from nitrate polluted area", Chemosphere, Vol. 62, pp. 559-564, 2006.
- [18] Rao, E.V.S.P., Puttana, K., "Nitrates, agriculture and environment", Curr. Sci., Vol. 79, pp. 1163-1168, 2000.
- [19] Bukowski, J., Somers, G., Bryanton, J., "Agricultural contamination of groundwater as a possible risk factor for growth restriction or prematurity", J. Occup. Environ. Med., Vol. 43, pp. 377-383, 2001.
- [20] Walthers, C.L., Carr, F.P., Dyke, C.S., Saxby, M.J., Smith, P.L., Walker, R., "Nitrite sources and nitrosamine formation *in vitro* and *in vitro*", Food Cosmet Toxicol., Vol. 17, PP. 473-479, 1979.

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

- [21] Hansen, P.R., Taxvig, C., Christiansen, S., Axelstad, M., Boberg, J., Kiersgaard, M.K., Nellemann, C., Hass, U., "Evaluation of Endocrine Disrupting Effects of Nitrate after In Utero Exposure in Rats and of Nitrate and Nitrite in the H295R and T-Screen Assay", *Toxicol. Sci.*, Vol. 108, pp. 437-444, 2009.
- [22] Sherman, G.J., Corey, P.N., "The effect of nitrate, nitrite and N-nitroso compounds in human health. A review", *Vet. Hum. Toxicol.*, Vol. 35, pp. 237-253, 1993.
- [23] World Health Organization, "Health hazards from nitrates in drinking water", *Environmental Health 1*, WHO, Copenhagen, 1985.
- [24] Fan, A.M., Willhite, C.C., Book, S.A., "Evaluation of the nitrate drinking water standard with reference to infant methemoglobinemia and potential reproductive toxicity", *Regul. Toxicol. Pharmacol.*, Vol. 7, pp. 135-148, 1987.
- [25] Dennis, M.J., Wilson, L.A., "Nitrates and Nitrites", In: Trugo, L., Finglas, P.M. (Eds.) *Encyclopedia of Food Sciences and Nutrition*. Central Science Laboratory., Sand Hutton, New York, pp. 4136-4141, 2003.
- [26] Addiscott, T.M., Benjamin, N., "Nitrate and human health", *Soil Use and Management*, Vol. 20, pp. 98-104, 2004.
- [27] Bensoltane, S., Messrer, L., Mustapha, Y., Berrebbah, H., Djekoun, M., Djebar, M.R., "Effects of acute and sub-chronic ammonium nitrate exposure on rat liver and blood tissues". *African J. Biotech.*, Vol. 5, pp. 749-754, 2006.
- [28] Kim, H.J., Chang, W. K., Kim, M.K., Lee, S.S., Choi, B.Y., "Dietary factors and gastric cancer in Korea: Case-control study", *Int. J. of Cancer*, Vol. 97, pp. 531-535, 2002.
- [29] Yarube, I.U., Halim, M.A., Osaka, M.E., Ayo, J.O., Olorunshola, K.V., "Antioxidant Vitamins C and E Alleviate the Toxicity Induced by Chronic Sodium Nitrate Administration on Sperm Count and Serum Testosterone Level in Wistar Rats", *Eur. J. Scientific Res.*, Vol. 25, pp. 35-41, 2009.
- [30] Brunning-Fann, C.S., Kaneene, J.B., "The effects of nitrate, nitrite and N-nitroso compounds on human health: A review", *Vet. Hum. Toxicol.*, Vol. 35, pp. 521-538, 1993.
- [31] Gruener, N., Shuval, H.I., "Methemoglobinemia induced by transplacental passage of nitrites in rats. Bull", *Environ. Contam. Toxicol.*, Vol. 9, pp. 44-48, 1973.
- [32] N. A. S., "The Health Effects of Nitrates, Nitrites, and N-Nitroso Compounds", National Academy of Sciences, Washington, DC., 1981.
- [33] Nestorova, J., Tsohev, I., Nikolov, I., Ivanova, N., "The effect of nitrates on sheep reproduction", *Zhivol Nauk.*, Vol. 34, pp. 149-151, 1997.
- [34] Pant, N., Srivastava, S.P., "Testicular and spermatotoxic effect of nitrate in mice", *Hum. Exp. Toxicol.*, Vol. 21, pp. 37-41, 2002.
- [35] Zraly, Z., Bendova, J., Svecova, D., Faldikova, L., Vezník, Z., Zajicova, A., "Effects of oral intake of nitrates on reproductive functions of bulls", *Vet Med (Praha)*, Vol. 42, pp. 345-354, 1997.
- [36] Panesar, N.S., Chan, K.W., "Decreased steroid hormone synthesis from inorganic nitrite and nitrate: Studies in vitro and in vivo", *Toxicol. Appl. Pharmacol.*, Vol. 169, pp. 222-230, 2000.
- [37] van Maanen, J.M., van Dijk, A., Mulder, K., de Baets, M.H., Menheere, P.C., van der Heide, D., Mertens, P.L., Kleinjans, J.C., "Consumption of drinking water with high nitrate levels causes hypertrophy of the thyroid", *Toxicol Lett.*, Vol. 72, pp. 365-374, 1994.
- [38] Barlow, N.J., McIntyre, B.S., Foster, P.M., "Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(n-butyl) phthalate". *Toxicol. Pathol.*, Vol. 32, pp. 79-90, 2004.
- [39] Borch, J., Ladefoged, O., Vinggaard, A.M., "Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats", *Reprod. Toxicol.*, Vol. 18, pp. 53-61, 2004.
- [40] Borch, J., Axelstad, M., Vinggaard, A.M., Dalgaard, M., "Diisobutyl phthalate has comparable anti-androgenic effects to di-n-butyl phthalate in fetal rat testis", *Toxicol. Lett.*, Vol. 163, pp. 183-190, 2006.
- [41] Carruthers, C.M., Foster, P.M., "Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate", *Birth Defects Res. B. Dev. Reprod. Toxicol.*, Vol. 74, pp. 277-285, 2005.
- [42] Ema, M., Miyawakiand, E., Kawashima, K., "Critical period for adverse effects on development of reproductive system in male offspring of rats given di-n-butyl phthalate during late pregnancy", *Toxicol. Lett.*, Vol. 111, pp. 271-278, 2000.
- [43] ma, M., Miyawaki, E., Hirose, A., Kamata, E., "Decreased anogenital distance and increased incidence of undescended testes in foetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate", *Reprod. Toxicol.*, Vol. 17, pp. 407-412, 2003.
- [44] Gray, L.E. Jr., Ostby, J., Furr, J., Price, M., Veeramachaneni, D.N., Parks, L., "Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat", *Toxicol. Sci.*, Vol. 58, pp. 350-365, 2000.
- [45] Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J., Gray, L.E. Jr., "The plasticizer diethylhexyl phthalate induces malformations by decreasing foetal testosterone synthesis during sexual differentiation in the male rat", *Toxicol. Sci.*, Vol. 58, pp. 339-349, 2000.
- [46] Srivastav, A., Chandra, A., Singh, M., Jamal, F., Rastogi, P., Rajendran, S.M., Bansode, F.W., Lakshmi, V., "Inhibition of hyaluronidase activity of human and rat spermatozoa in vitro and antispermatogenic activity in vivo by *Terminalia chebula*, a flavonoid rich plant", *Reprod. Toxicol.*, Vol. 29, pp. 214-224, 2010.
- [47] Abercrombie, M., "Estimation of nuclear population from microtome sections", *Anat. Rec.*, Vol. 94, pp. 238-248, 1946.
- [48] Amann, R.P., Johnson, L., Thomson, D.L., Pickett, B.W., "Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the rhesus monkey", *Biol. Reprod.*, Vol. 15, pp. 586-592, 1976.
- [49] Amann, R.P., Lambiase, Jr, J.T., "The male rabbit. III. Determination of daily sperm production by means of testicular homogenates", *J. Anim. Sci.*, Vol. 28, pp. 369-374, 1969.
- [50] Robb, G.W., Amann, R.P., Killian, G.J., "Daily sperm production and epididymal sperm reserves of pubertal and adult rats", *J. Reprod. Fertil.*, Vol. 54, pp. 103-107, 1978.
- [51] Talalay, P., "Hydroxysteroid dehydrogenase, in: Colowick, S.P., Kaplan, N.O. (Eds.), *Methods in Enzymology*". Academic Press, New York, pp. 512-516, 1962.

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 7, July 2017

- [52] Jarabak, J., Adams, J.A., Williams-Ashman, H.G., Talalay, P., "Purification of a 17 β -hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function", J. Biol. Chem., Vol. 237, pp. 345-357, 1962.
- [53] Bradford, M., "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding", Anal Biochem., Vol. 72, pp. 248-254, 1976.
- [54] Pant, N., Shanker, R., Srivastava, S.P., "In utero and lactational exposure of carbofuran to rats: effect on testes and sperm", Hum. Exp. Toxicol., Vol. 16, pp. 267-272, 1997.
- [55] Misro, M.M., Ganguli, A., Das, R.P., "Is Testosterone Essential for Maintenance of Normal Morphology in Immature Rat Leydig Cell?", Int. J. Androl., Vol. 16, pp. 221-226, 1993.
- [56] Russell, L.D., Corbin, T.J., Ren, H.P., Amador, A., Bartke, A., Ghosh, S., "Structural changes in rat Leydig cells post hypophysectomy: a morphometric and endocrine study", Endocrinol., Vol. 131, pp. 498-508, 1992.
- [57] Shan, L.X., Hardy, M.P., "Developmental changes levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells", Endocrinol., Vol. 131, pp. 1107-1114, 1992.
- [58] Kerr, J.B., Millar, M., Maddocks, S., Sharp, R.M., "Stage-dependent changes in spermatogenesis and Sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone", Anat. Rec., Vol. 235, pp. 547-559, 1999.
- [59] Tähkä, K. M., "A histochemical study on the effects of photoperiod on gonadal and adrenal function in the male bank vole (*Clethrionomys glareolus*)", J. Reprod. Fertil., Vol. 54, pp. 57-66, 1978.
- [60] Chapman, J.C., Polanco, J.R., Min, S., Michael, S.D., "Mitochondrial 3 beta-hydroxysteroid dehydrogenase (HSD) is essential for the synthesis of progesterone by corpora lutea: An hypothesis", Reprod. Biol. Endocrinol., Vol. 3, pp. 11, 2005.
- [61] Prisco, M., Liguoro, A., Ricchiari, L., Giudice, G.D., Angelini, F., Andreuccetti, P., "Immunolocalization of 3 β -HSD and 17 β -HSD in the testis of the spotted ray *Torpedo marmorata*", Gen. Comp. Endocrinol., Vol. 155, pp. 157-163, 2008.
- [62] Srivastava, S., Singh, G.B., Srivastava, S.P., Seth, P.K., "Testicular toxicity of di-n-butylphthalate in adult rats effect on marker enzymes of spermatogenesis", Ind. J. Exp. Biol., Vol. 28, pp. 67-70, 1990.
- [63] Srivastava, S., Srivastava, S.P., "Effect of di (2-ethylhexyl) phthalate on 17 beta-hydroxysteroid dehydrogenase activity in testis of rat", Toxicol. Lett., Vol. 57, pp. 235-239, 1991.
- [64] Diaz, J., Fernandez-Arjona, M., Perandones, C., Serrano, E., Frau, C., Cortes, I., Luque, A., Carbonell, L.F., "Reference intervals for biochemistry parameters for evaluation of oxidative stress in human sperm", Clin. Chem., Vol. 44, pp. 2197-2199, 1998.
- [65] O'Donnell, L., McLachlan, R.I., Wreford, N.G., Robertson, D.M., "Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle", Endocrinol., Vol. 135, pp. 2608-2614, 1994.
- [66] Veeramachaneni, D.N.R., Palmer, J.S., Amann, R.P., "Long-term effects on male reproduction of early exposure to common chemical contaminants in drinking water", Hum Reprod., Vol. 16, pp. 979-987, 2001.
- [67] Gupta, R.S., Bhatnager, A.K., Joshi, Y.C., Sharma, M.C., Khushalani, V., Kachhawa, J.B., "Induction of antifertility with lupeol acetate in male albino rats", Pharmacol., Vol. 75, pp. 57-62, 2005.
- [68] Zhao, D., Zhu, C., Sun, S., Yu, H., Zhang, L., Pan, W., Zhang, X., Yu, H., Gu, J., Cheng, S., "Toxicity of pharmaceutical wastewater on male reproductive system of *Mus musculus*", Toxicol Indust Health, Vol. 23, pp. 47-54, 2007.
- [69] Viroj, W., "Benzene Exposure and Spermatotoxicity", Sexuality and Disability, Vol. 24, pp. 179-182, 2006.
- [70] Xiao, G., Pan, C., Cai, Y., Lin, H., Fu, Z., "Effect of benzene, toluene, xylene on the semen quality and function of accessory gonad of exposed workers", Industrial Health, Vol. 39, pp. 206-210, 2001.
- [71] Bansode, F.W., Dwivedi, A.K., Maikhuri, J.P., Chowdhury, S.R., "Quantitative analysis of spermatogenesis in rat made azoospermic with Compound CDRI 84/35", Contraception, Vol. 58, pp. 175-182, 1998.
- [72] Bansode, F.W., Dwivedi, A.K., Chowdhury, S.R., "Effect of compound CDRI84/35 and synthetic estrogen on the seminiferous epithelium of immature rat", Endocr. Res., Vol. 28, pp.103-117, 2002.
- [73] Kishta, O., Adeeko, A., Li, D., Luu, T., Brawer, J.R., Morales, C., Hermo, L., Robaire, B., Hales, B.F., Barthemy, J., Cyr, D.G., Yrasler, J.M., "In utero exposure to tributyltin chloride differentially alters male and female fetal gonad morphology and gene expression profiles in the Sprague-Dawley rat", Reprod. Toxicol., Vol. 23, pp. 1-11, 2007.
- [74] Lemasters, G.K., Olsen, D.M., Yiin, J.H., Lockey, J.E., Shukla, R., Selevan, S.G., Schrader, S.M., Toth, G.P., Evenson, D.P., Huszar, G.B., "Male reproductive effects of solvent and fuel exposure during aircraft maintenance", Reprod. Toxicol., Vol. 13, pp. 155-166, 1999.
- [75] McLachlan, R.I., Wreford, N.G., Meachem, S.J., De Krestser, D.M., Robertson, D.M., "Effects of Testosterone on Spermatogenic Cell Populations in the Adult Rat", Biol. Reprod., Vol. 51, pp. 945-955, 1994.
- [76] Stangherlin, E.C., Favero, A.M., Weis, S.N., Zeni, G., Rocha, J.B., Nogueira, C.W., "Assessment of reproductive toxicity in male rats following acute and sub-chronic exposures to diphenyl diselenide and diphenyl ditelluride", Food Chem Toxicol., Vol. 44, pp. 662-669, 2006.
- [77] vom Saal, F.S., Akingbemi, B.T., Belcher, S.M., Birnbaum, L.S., Crain, D.A., Eriksen, M., Farabolini, F., Guillette, L.J.Jr., Hauser, R., Heindel, J.J., Ho, S.M., Hunt, P.A., Iguchi, T., Jobling, S., Kanno, J., Keri, R.A., Knudsen, K.E., Laufer, H., LeBlanc, G.A., Marcus, M., McLachlan, J.A., Myers, J.P., Nadal, A., Newbold, R.R., Olea, N., Prins, G.S., Richter, C.A., Rubin, B.S., Sonnenschein, C., Soto, A.M., Talsness, C.E., Vandenbergh, J.G., Vandenberg, L.N., Walsler-Kuntz, D.R., Watson, C.S., Welshons, W.V., Wetherill, Y., Zoeller, R.T., "Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure", Reprod. Toxicol., Vol. 24, pp.131-138, 2007.
- [78] Shi, Y., Song, Y., Wang, Y., Liang, X., Hu, Y., Guan, X., Cheng, J., Yang, K., "p,p'-DDE Induces Apoptosis of Rat Sertoli Cells via a FasL-Dependent Pathway", J. Biomed. Biotechnol., Vol. 2009, pp. 1-11, 2009.