Benzene-induced histopathological changes and germ cell population dynamics in testes of Sprague Dawley rats

Author Details

R.K. Singh
(Corresponding author)
Division of Toxicology, Central Drug Research Institute, Chattar Manzil, Lucknow - 226 001, India
e-mail: rktox@yahoo.com

F.W. Bansode
Division of Endocrinology, Central Drug Research Institute, Chattar Manzil, Lucknow - 226 001, India

Abstract

Benzene has been considered as an occupational hematotoxin and leukemogen. The present study was conducted to determine the effects of oral administration of benzene on reproductive organs and testicular spermatogenesis in rats. Adult rats were divided into three weight matched groups (Gr. I-III) containing 6 each. Gr. I rats received vehicle only and served as control. Rats in Gr. II and III were fed orally with 0.5 and 1 ml kg\(^{-1}\) dose of benzene for 14 and 9 days, respectively and autopsy was done on 15\(^{th}\) and 10\(^{th}\) day. Food and water intake and gross behavioral changes were recorded daily during the entire treatment. Results showed no significant change in reproductive organ weights viz. testis, epididymis and ventral prostate in benzene-treated (0.5 or 1 ml kg\(^{-1}\)) rats than that in controls. But, caused a significant decrease (p<0.005) in weights of seminal vesicles in rats treated with both 0.5 and 1 ml kg\(^{-1}\) doses compared to control. In contrast, at higher dose (1 ml kg\(^{-1}\)) of benzene, significant (p<0.001) decline in body weight and 100% mortality was observed on day 10 of autopsy. In treated rats, testicular cytotoxicity was marked by multinucleated giant cells formation, cytoplasmic vacuolization, pyknosis of nuclei, chromatolysis, desquamation and dissolution of germ cells in tubular lumen. The quantitative analysis of spermatogenesis showed a significant (p<0.001) decrease in number of A-spermatogonia (in 1 ml kg\(^{-1}\) dose only), primary spermatocytes (non-pachytene and pachytene) and spermatids (round and elongated) in treated as compared to control rats. The diameters of testicular tubules and Leydig cells nuclei were also significantly (p<0.001) reduced in treated rats. A steady loss in food and water intake recorded and signs of ill health were observed in treated (0.5 or 1 ml kg\(^{-1}\)) rats.

Results of the study indicated antitesticular/antispermatogenic effects of benzene at 0.5 and 1 ml kg\(^{-1}\) dose in rats.

Key words

Spermatogenesis, Population dynamics, Leydig cell, Histology, Benzene

Introduction

Benzene is an important and widely used industrial solvent for many substances. It has been considered as one of the most common polluting factors of chemical origin which is mostly present in mineral oil, natural gas, motor vehicle emission and tobacco smoke (Paustenbach et al., 1993; Schlosser et al., 1993; Wallace et al., 1997; Brugnone et al., 1999; Gordon et al., 2002). Epidemiological studies have shown an increase of leukemia and aplastic anemia in workers exposed to benzene (Aksoy, 1991; Duarte-Davidson et al., 2001; Van Wijngaarden and Stewart, 2003; Glass et al., 2010; Khalade et al., 2010). It is well known that benzene and its metabolites induce hematopoietic toxicity, characterized by decrease in bone marrow (BM)cellularity and the induction of leucopenia, aplastic anaemia, and leukemia (Ning et al., 1991; Tompa et al., 1994; Ross, 1996; Williams and Paustenbach, 2003; Mellert et al., 2007; Atkinson, 2009; Lovreglio et al., 2010). Several hypotheses regarding benzene-induced toxicity are based upon in vivo studies with limited parameters of hematopoiesis which includes bone marrow cells, progenitor cells, differentiating hematopoietic cells and blood parameters (Irons and Stillman, 1996; Farris et al., 1997a). However, mechanism of action of benzene-induced effects such as cytotoxicity, apoptosis, mutagenesis and cell replication in bone marrow and blood cells as well as in spleen, lung and liver cells in both a dose-dependent and time-dependent manner are based upon in vitro studies (Snyder et al., 1993; Chen et al., 1994; Snyder and Kaif, 1994; Farris et al., 1997a).
The various pathways including metabolism, growth factor regulation, oxidative stress associated with increased levels of ROS, DNA and RNA damage, cell cycle regulation and programmed cell death are involved in the mechanism of benzene-induced toxicity and leukemogenicity, and cDNA micro array provided valuable information regarding its mechanism of action recently (Tuo et al., 1996; Yoon et al., 2003; Emara and El-Bahrawy, 2008; Lau et al., 2009; Yi et al., 2009; Badham et al., 2010; Badham and Winn, 2010; Bi et al., 2010; Hirabayashi and Inoue, 2010; Manini et al., 2010; Yang and Zhou, 2010).

In males, recent evidences have shown antifertility effects of benzene which included the sperm characteristics, acrosin activity, LDH enzyme activity and accessory sex organ function in the workers exposed to benzene and other toxicants also (Xiao et al., 1999; 2001). Testicular antispermatogenic effects and inhibition of accessory gland function have been reported with benzene or its isolated compound, Lupeol acetate in rat (Gupta et al. 2005), rabbit (Veeramachaneni et al., 2001) and Mus musculus (Zhao et al., 2007). In fishes too, alkyl benzene sulphonate has been shown to induce testicular cytotoxicity (Kumar et al., 2007). In vitro studies have shown that β-benzene hexachloride induces apoptosis in rat Sertoli cells through generation of reactive oxygen species and activation of JNKs and FasL and activation of caspase cascade (Shi et al., 2009a).

Present study was undertaken to evaluate 1. Weight change in the reproductive organs, 2. Histology of testis, 3. Population dynamics of germ cells and Sertoli cells, and 4. Leydig cells morphology in adult rats administered with benzene (0.5 and 1 ml kg⁻¹, p.o.) for 14 and 9 days, respectively.

**Materials and Methods**

Adult male Sprague Dawley rats (150-170 g body weight) obtained from the Institute’s breeding colony were acclimatized to housing conditions for 1 week prior to the experiment. They were maintained under uniform husbandry conditions (22±3°C, 12 hr light: 12 hr dark cycle). Food (Standard pellets) was obtained from Hindustan Lever Ltd., Bangalore, India. All rats were handled in accordance with the standard guide (OECD Guidelines, 2003) for the care and use of laboratory animals.

In experimental design, a total of 18 adult male rats were divided into three weight-matched groups (Gr.I-III) containing six animals each and housed in autoclaved steel cages in laboratory conditions. Rats in Gr. I were given vehicle (distilled water) only and served as control. Rats in Gr. II and III were fed orally by using feeding cannula with two different doses i.e. 0.5 and 1 ml kg⁻¹ body weight of benzene for 14 and 9 days, respectively. Food and water intake, and behavioral changes were recorded daily. For food and water consumption, in the beginning of the experiment the weighed amount of pellet diet and measured volume of water was kept for each rat in individual cages separately. Then animals were allowed to free access to drinking water and food. Following next day after 24 hr, the amount of food and water left was measured and subtracted from initial amount provided. The resultant “difference” between initial and final food and water intake was calculated and recorded during the entire treatment period. The physical appearances and behavioral activities of the rats were observed daily for 1 hr after the administration of benzene, included the salivation, lacrimation, ptosis, exophthalmos, arousal response, spontaneous motor activity, posture/position, gait, ataxia, tremor, convulsions, straub’s tail, abnormal stereotypy, catalepsy, righting reflex, defecation and urination etc.

Autopsy of control (Gr.I) and benzene-treated (Gr. II, 0.5 ml kg⁻¹) rats was done on day 15 by anaeasthetizing with solvent anaesthetizing ether. Autopsy of Gr. III rats treated with 1ml kg⁻¹ dose of benzene was done in the morning on 10⁶ day due to 100% mortality observed after 9 days treatment. The reproductive organs viz. testes, epididymis and accessory sex organs (ventral prostate and seminal vesicles) were dissected out surgically under semi sterile conditions, rinsed immediately in chilled saline, freed from connective tissues/ blood clots, then weighed and fixed in Bouin’s fluid (24 hr) for histology purpose. The whole process was carried out under the guidelines laid down by Institute’s Animal Ethics Committee.

Quantitation of testicular germ cell population dynamics was carried out on the basis of nuclear morphology of the germ and Sertoli cells in control and treated (0.5 and 1 ml kg⁻¹) groups of rats, where the number of germ cells 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 to zygotene) and pachytene spermatocytes, and round and elongated spermatids were assessed in each group of 6 rats at 400X magnification under Olympus trinocular microscope(Olympus, BX 51, Japan). Cells counts estimated as the number of germ cells as well as Sertoli cell number per cross section of the round seminiferous tubule and corrected by Abercrombie’s formula (Abercrombie, 1946). Measurements (µm) of tubular diameter (15-20 tubules each rat with total of 100 tubules group⁻¹) and the nuclear diameter of Leydig cells (20 Leydig cells each rat with total of 100-150 cells group⁻¹) were taken 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).

Statistical analysis was done by Students’ t test for significance level between control and treated groups. Values were expressed as mean ± standard error (SE).

**Results and Discussion**

Body weights (220.67±5.3 vs. 211.14±9.3, Mean±SE, control vs. treated) did not show any significant change in rats (Gr. II) treated with 0.5 ml kg⁻¹ benzene for 14 days as compared to control (Gr.I) rats. In contrast, rats in Gr. III treated with 1ml kg⁻¹
Fig. 1: Histology of testis from control (A) and benzene-treated (B-D) rats (H&E X 400). A = Normal germ cell types, e.g. Spermatogonia, primary spermatocytes and round and elongated spermatids, B and C = Seminiferous epithelium exhibiting cytoplasmic vacuolization (\(\uparrow\)), pycnotic nuclei (\(\Delta\)), chromatolysis (\(\downarrow\)) and desquamated germ cells (Asterisk) in tubular lumen of rats treated with benzene at 0.5 ml kg\(^{-1}\) dose for 14 days and 1ml kg\(^{-1}\) dose (D) for 9 days. A, B- Spermatogonia, Z- Zygotene spermatocytes, P- Pachytene spermatocytes, R- Round spermatids, and E- Elongated spermatids, L- Lumen, LC- Leydig cells, SC- Sertoli cells.

Fig. 2: Leydig cell (LC) morphology in control (A) and benzene-treated (B-D) rats [H&E X 600] A= Normal Leydig cells in control rat, B and C = Note the reduced size and attenuated profile of Leydig cells in benzene-treated rats at 0.5 ml kg\(^{-1}\) dose for 14 days and at 1ml kg\(^{-1}\) dose (D) for 9 days, as compared to in Fig. 2A.
In testes of benzene-treated (0.5 or 1 ml kg\(^{-1}\)) rats, showed non-significant reduction in weights of reproductive organs viz. testes (1.65±0.23 vs. 1.35±0.19), epididymis (0.51±0.07 vs. 0.41±0.05) and ventral prostate gland (0.322±0.0679 vs. 0.245±0.011) have shown no significant change (reduction) at 0.5 ml kg\(^{-1}\) dose of benzene. But, caused a significant decline (p<0.005) in the weight of accessory sex gland viz. seminal vesicles (0.572±0.054 vs. 0.343±0.035) in treated as compared to control rats (Table 1). Similarly, rats in Gr. III treated with higher dose (1ml kg\(^{-1}\)) of benzene, showed non-significant reduction in weights of testes (1.65±0.23 vs. 1.55±0.26), epididymis (0.51±0.077 vs. 0.37±0.11) and ventral prostate (0.322±0.0679 vs. 0.287±0.047) but, caused significant decline (p<0.001) in weight of seminal vesicles (0.572±0.054 vs. 0.438±0.105) than that in controls (Table 1).

The quantitative analysis of germ cells population viz. non-pachytene spermatocytes (9.002±0.8124 vs. 4.664±1.481, control vs. treated, p<0.02), pachytene spermatocytes (7.113±0.618 vs. 0.892±0.283, p<0.001), round spermatids (22.276±2.375 vs. 1.672±0.336, p<0.001) and elongated spermatids (17.81±1.902 vs. 0.654±0.274, p<0.001) showed significant decrease in benzene (0.5 ml kg\(^{-1}\) dose)-treated as compared to control rats. However, number of A-spermatogonia (1.424±0.1012 vs. 1.395±0.1424 and Sertoli cell number (3.584±0.1945 vs. 4.048±0.270) did not change significantly in treated (0.5 ml kg\(^{-1}\)) rats (Table 2). While in rats administered 1 ml kg\(^{-1}\) dose of benzene showed pronounced decrease in number of primary spermatocytes (non pachytene and pachytene) and spermatids (round and elongated) including inhibition (p<0.02) of A-spermatogonia compared to controls (Table 2).

In control rats, testicular histology revealed normal spermatogenesis depicting all the germ cells types, viz. spermatogonia, primary spermatocytes (non-pachytene and pachytene) and spermatids (round and elongated) in the seminiferous epithelium. The Sertoli and interstitial Leydig cells also showed normal morphology (Fig. 1A and 2A). In testes of benzene-treated (0.5 or 1 ml kg\(^{-1}\)) rats, degenerative changes in the seminiferous epithelium were characterized by the giant cell formation, cytoplasmic vacuolization, pycnosis, chromatolysis, desquamation and dissolution of germ cells in tubular lumen. The Sertoli cells showed normal morphology in treated rats (Fig. 1B, C and D). But, Leydig cells showed elongated appearance/attenuated profile with reduced size (Fig. 2B, C and D). Testicular tubular diameter (538.50±6.758 vs. 412.98±10.776; 385.35±5.525) and nuclear diameter of Leydig cells (13.47±0.18 vs. 10.40±0.19; 8.53±0.25) were also significantly reduced (p<0.001) in treated (0.5 and 1ml kg\(^{-1}\)) as compared to control group of rats. The degenerative changes in seminiferous tubules were more pronounced in rats treated with higher (1 ml kg\(^{-1}\)) dose of benzene (Table 2).
treatment at 0.5 mg kg\(^{-1}\) dose and from day 4 onwards at 1 mg kg\(^{-1}\) dose as compared to corresponding control rats (Fig. 3). Water intake record showed differential pattern in treated rats. The amount of water intake was decreased significantly on days 8 (p<0.02), 10 (p<0.05) and 11 (p<0.01) of benzene treatment at 0.5 mg kg\(^{-1}\) dose as compared to corresponding controls. In contrast, at its higher (1 mg kg\(^{-1}\)) dose, a significant decrease in water intake was observed from days 4-9 of treatment (Fig. 4). There were some sign of ill health like morbidity, dysentery and gait in posture during the treatment with 0.5 ml kg\(^{-1}\) dose of benzene. While, in rats administered at its higher dose (1 ml kg\(^{-1}\)) showed tiredness, nausea, depression, balance disorder and nose bleeding.

The present quantitative analysis of spermatogenesis indicated significant inhibition of primary spermatocytes viz. non-pachytene (p<0.02) and pachytene (p<0.001) and spermatids (round and elongated in steps 1-19 of spermiogenesis, p<0.001) in both 0.5 or 1 ml kg\(^{-1}\) doses of benzene for 14 and 9 days treatment as compared to control rats (Table 2). The suppression of spermatogenic germ cells was also accompanied by extensive cytotoxicity in seminiferous epithelium depicting cytoplasmic vacuolization, giant cell formation, chromatolysis, pyknosis of nuclei, desquamation and dissolution of germ cells in tubule lumen in treated (0.5 or 1 mg kg\(^{-1}\)) rats. These cytotoxic effects were more pronounced at its higher dose (1 mg kg\(^{-1}\)) after 9 days of treatment. Further, benzene treatment caused significant decrease (p<0.001) in testicular tubular diameters at both doses (0.5 or 1 ml kg\(^{-1}\)) compared to controls. Results of the present study can be correlated with the previously reported studies that indicate the depletion of primary and secondary spermatocytes and spermatids (in step 19) with an active compound of benzene, lupeol acetate at 10 mg kg\(^{-1}\) in rats (Gupta et al., 2005) and intragastric perfusion of waste water (contaminant of benzene) in Mus musculus (Zhao et al., 2007). In humans too, benzene exposure has been shown to cause spermatozoa toxicity including inhibition of semen quality, sperm counts and sperm motility (Viroj, 2006; Xiao et al., 1999; 2001). Flow cytometric (FCM) analysis of DNA content in mouse testicular monolayer cell suspensions exposed per oral suspension to 0, 1, 2, 4, 6 and 7 ml kg\(^{-1}\) body weight of benzene for 7, 14, 21, 28, and 70 days have shown decline in the relative percentages of some cell subpopulations (tetraploid and haploid cells), indicating the occurrence of some cytotoxic damage to differentiating spermatogonia without affecting testis weight (Spano et al., 1989). Results of the present study did not show any significant change in number of spermatogonia and Sertoli cells at lower dose (0.5 mg kg\(^{-1}\)) but, caused significant inhibition (p<0.02) in spermatogonial number at its higher dose (1 ml kg\(^{-1}\)) without affecting testicular weight. The short term exposure to benzene has been shown to cause some changes (increases then decreases) in LDH, ALP, ALT, AST, and PK activity and estradiol, testosterone concentrations in different tissues of rats (Egemen et al., 2003). Increased activity of ACP and decreased ALP activity in testis has also been correlated with gross necrosis and dysarchitecture of testis (Trivedi et al., 2001). Maximum testicular cytotoxicity with shorter duration at higher concentration of benzene than in its lower concentration for longer duration has been reported previously (Kumar et al., 2007). In rabbit, the mixture of arsenic, chromium, lead, benzene, chloroform, phenol and trichloroethylene to the individual males until 15 weeks, has shown to cause a decrease in total spermatozoa/ejaculates, daily sperm production, acrosomal dysgenesis, nuclear malformations, mating desirability, sperm quality and Leydig cell function associated with decreased serum concentrations of LH and testosterone secretion (Veeramachaneni et al., 2001). Similarly, antitesticular / antispermatogenic effects in rodents have also been reported with other pollutants/ chemical agents as well as estrogens/antiestrogens/ androgens (Bansode et al., 1998; 2002; Kishita et al., 2007; Lemasters et al., 1999; McLachlan et al., 1994; Misro et al., 1993; Stangherlin et al., 2006; vom Saal et al., 2007; Shi et al., 2009b).

Our results also showed significant inhibition of androgenic function evidenced by reduced size and morphology of Leydig cells and significant decline (p<0.005) in accessory sex organ (seminal vesicle) weights in benzene-treated (0.5 or 1 ml kg\(^{-1}\)) rats compared to control. Earlier studies on testosterone deprivation have shown suppression of plasma LH, nuclear morphology of Leydig cells and germ cells. Testosterone supplementation restored normal function of Leydig cells and spermatogenesis which emphasizes the testosterone dependent cellular sensitivity of Leydig

**Fig. 3:** Food intake record in control and benzene (0.5 and 1 ml kg\(^{-1}\)) treated rats. (Values mean±SE, n = 6 animals, Significance between control and treated, a = p<0.05; b = p<0.005 and c = p<0.001)

**Fig. 4:** Water intake record in control and benzene (0.5 and 1 ml kg\(^{-1}\)) treated rats. (Values mean±SE, n = 6 animals, Significance between control and treated, a = p<0.05; b = p<0.02, c = p<0.01 and d = p<0.001)
The authors are thankful to Ms. T. Firdaus, Anju Rani (Trainee), Mr. S. K. Srivastava and Mr. Nand Pal Yadav from Endocrinology and Toxicology Division for technical assistance in the preparation of histological slides and experimental work respectively. This study was supported by grants from the Ministry of Health and Family Welfare, Government of India.

Acknowledgments

The authors are thankful to Ms. T. Firdaus, Anju Rani (Trainee), Mr. S. K. Srivastava and Mr. Nand Pal Yadav from Endocrinology and Toxicology Division for technical assistance in the preparation of histological slides and experimental work respectively. This study was supported by grants from the Ministry of Health and Family Welfare, Government of India.

References


Journal of Environmental Biology  November 2011  

R.K. Singh and F.W. Bansode

cells (Misro _et al._, 1993; Russell _et al._, 1992; Shan _et al._, 1992; Kerr _et al._, 1993). Our results too show an evidence of depletion of androgen-dependent meiotic primary spermatocytes and postmeiotic spermatids in association with decreased Leydig cell diameter (at 0.5 or 1 ml kg^(-1)) and spermatogonia (at 1 ml kg^(-1) dose only) with benzene treatment and may indicate testosterone inhibition (O’Donnell _et al._, 1994). Further, there was an inhibition of food consumption and differential pattern of water intake in treated rats, and prominent behavioral changes caused at higher dose may lead to mortality of rats affecting inhibition of body weight gain at higher dose (1 ml kg^(-1)) of benzene. Inhibition of body weight gain has been also reported with n-butyl benzene treatment previously by Izumi _et al._ (2005).

The present study demonstrates the antitesticular / antispermatogenic effects of benzene at 0.5 or 1 ml kg^(-1) doses for 14 and 9 days in rats. Inhibition of testicular spermatogenesis by benzene treatment exhibits an evidence of depletion of androgen-dependent meiotic primary spermatocytes (non-pachytene and pachytene and pachytene) and postmeiotic spermatids (round and elongated) germ cells. It was also associated with decreased testicular tubular diameter, nuclear diameter of Leydig cells and accessory sex organ (seminal vesicle) weight may indicate anti-androgenic effects of benzene at 0.5 or 1 ml kg^(-1) dose level in rats.
Testicular toxicity of benzene


Shan, L.X. and M.P. Hardy: Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells. Endocrinol., 131, 1107-1114 (1992).


