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Lead aggravates the diabetic-induced reproductive toxicity in male Wistar rats

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SUMMARY

Diabetes, an unresolved metabolic disorder and lead contamination are prevalent problems in the contemporary society. Previously, we have reported that either diabetes or lead exposure resulted in reproductive toxicity in male Wistar rats. The aim of this study was to evaluate whether diabetic rats exposed to lead demonstrate a higher degree of reproductive toxicity when compared with lead-exposed control rats. Diabetes was induced by injecting single dose of streptozotocin (50 mg/kg body weight). Control and diabetic rats were exposed to lead at a concentration of 819 mg/L (0.15% lead acetate) through drinking water for a period of 30 days and assessed for reproductive and oxidative end points. The relative weights of testes, epididymis, seminal vesicles, and prostate gland were significantly decreased in diabetic rats. Daily sperm production, epididymal sperm count, motile, viable and HOS-tail swelled sperms, serum testosterone levels and testicular 3 β- and 17 β-hydroxysteroid dehydrogenase activity levels were significantly decreased in diabetic rats. Significant reduction in testicular and epididymal antioxidant enzyme activity levels and glutathione levels were observed in diabetic rats with an elevation in levels of superoxide anions, hydrogen peroxides, lipid peroxidation. Significant reduction in the number of implantations associated with elevated pre- and post-implantation losses was observed in females mated with diabetic males. Mild histopathological malformations were observed in the testis of the diabetic rats. Similar reproductive and oxidative toxicity was observed in lead-exposed control rats. Further, lead exposed diabetic rats showed additional deterioration in reproductive end points and noteworthy elevation in oxidative toxicity suggesting that treatment with lead exacerbates reproductive toxicity in streptozotocin-induced diabetic rats.

Keywords: Streptozotocin, lead, oxidative stress, spermatogenesis, steroidogenesis.

Introduction

Diabetes mellitus is a metabolic disorder characterized by high blood sugar, resulting from insufficient production of insulin by the pancreas or ineffective utilization of insulin by the body or both.[1] According to 2011 estimates, there are 366 million people living with diabetes, and it has been predicted that this will grow to 552 million by 2030.[2] Diabetic complications cause noticeable sexual complications like erectile dysfunction, retrograde ejaculation, and changes in testosterone levels.[3,4] It has been estimated that about 35-75% of men with diabetes will experience at least a minimum degree of sexual impotence during their lifetime.[5] The possible cause of sexual dysfunction in diabetic men may be impairment of nerves, blood vessels, and muscle function. Besides this, over production of reactive oxygen species (ROS)[6] and compromised androgen production[4] in diabetic males also stands as a prominent reason. The surplus amounts of ROS affect sperm DNA, sperm function, testicular metabolite levels, and fusion events associated with fertilization, leading to infertility.[7]

Lead (Pb) toxicity remains a significant public health problem because of its global pervasiveness, and due to its bio-accumulative, non-biodegradable and immutable properties. It is well established that Pb affects both testicular[8] and epididymal functions.[9] Our earlier study also reported that Pb-exposure alters spermatogenesis and steroidogenesis thereby affects fertility efficiency in male rats.[10] Experimental evidence also suggests that cellular damage mediated by free radicals can be involved in the pathology associated with Pb intoxication.[11] It is well established that Pb alters the lipid metabolism and enhances lipid peroxidation (LPx) and increases free radicals through the inhibition of antioxidant enzymes in testis and epididymis.[12,13] Oxidative stress condition prevails when there is an imbalance between reactive oxygen species (ROS) production and endogenous antioxidantsand antioxidant enzymatic levels. Physiological

levels of ROS such as superoxide, hydroxyl and peroxyl radicals are required for normal functioning and at high concentrations these are toxic to the functioning of the cells. Spermatozoa being rich in polyunsaturated fatty acids are vulnerable targets to damage caused by ROS. The excessive generation of free radicals has also been identified as a major contributing factor in the etiology of male infertility by triggering sperm dysfunction.

Although a large number of studies have reported separately on the deleterious effects of Pb or diabetes on reproduction, there have been no reports dealing particularly with the Pb-induced detrimental effects on reproduction in diabetic male rats. Co-exposure to multiple stresses is widely present in the real world since single stress exposure to human body is rare, and combination of stresses might synergistically affect human health. Our earlier studies focused on combinative toxicity of restraint stress and lead[14], restraint stress and alcohol[15], alcohol and diabetes[16], and benzo(a)pyrene and alcohol[17] on reproduction in rats, and additive or synergistic interactions have been discovered in some combinations. We have extended our studies to examine the effect of Pb on oxidative status in the testis and epididymis and sexual dysfunction in streptozotocin (STZ)-induced diabetic rats. Other aspects of this study have been published or submitted for publication. The effects of Pb on reproductive and developmental toxicity in male rats were published.[10] Diabetic-induced oxidative toxicity and sexual dysfunction in adult male Wistar rats have also been published.[16] The effects of peri-natal exposure to elevated sugar levels on developmental, behavioral and reproductive parameters have been submitted for publication.

Materialsand methods

Procurement and maintenance of animals

Male Wistar albino rats with a body weight of 190±10 g (90 days old) were purchased from authorized vendor (Sri Ragavendra Enterprises, Bengaluru, India). Rats were housed (four per cage) in clean polypropylene cages (18" x 10" x 8") with paddy husk bedding in the animal house facility and maintained at 23 ± 2°C and relative humidity 60 ±10% with a 12 h light/day cycle. Standard rodent chow (obtained from SaiDurga Agencies, Bengaluru, India) and water were made available *ad libitum*. The experiments were carried out in accordance with guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.[18] The protocol and animal use were approved by Institutional Animal Ethical Committee (Regd. No. 438/01/a/CPCSEA/dt.17.07.2001) in its resolution No: 57/2012/(i)/a/CPCSEA/IAEC/SVU/PSR-KPR dt.08-07-2012. The study is planned and organized as completely double blind.

Chemicals

STZ (≥98% purity), androstenedione (≥98% purity), dihydroepiandrosterone (98% purity), NAD (≥98% purity) and NADPH (97% purity), INT (95% purity), horse radish peroxidase (>90%), glutathione reductase (≥90% purity), reduced glutathione (≥98% purity), and oxidized glutathione (≥98% purity) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Lead acetate (≥99.5% purity), dimethyl sulfoxide (DMSO) (purity ≥99%), malondialdehyde (MDA) (purity ≥95%), pyrogallol (purity ≥99%) were purchased from Merck, Darmstadt, Germany. All other chemicals used in study were purchased from HIMEDIA (Bangalore, India).

Induction of diabetes mellitus

Diabetes was induced in healthy male Wistar rats by a single intraperitoneal injection of freshly prepared STZ (50 mg/kg body weight) dissolved in ice cold citrate buffer (0.1M, pH 4.5).

Control rats received vehicle only. The rats were allowed to drink 5% glucose solution overnight after injection, to overcome the drug-induced hypoglycemia. After 72 h of STZ injection, animals with above 300 mg/dl of fasting blood glucose were considered as diabetic rats and used for the experiment.

Experimental design and necropsy

In this study the animals were divided into four groups: Group 1: Control rats, intra-peritoneally injected with 100 µL of cold citrate buffer (0.1 M, pH 4.5); Group 2: Pb-exposed rats; Group 3: Diabetic control rats; Group 4: Diabetic rats exposed to Pb. The animals in groups 2 and 4 were allowed *ad libitum* access to tap water containing 819 mg/L lead (0.15% lead acetate) for 30 days. The concentration of Pb and the duration of treatment period was selected on the basis of our earlier studies.[10] A stock solution of 1% dissolved in distilled water; immediately prior use, it was diluted with filtered tap water to the desired concentration. At the end of the experiment, all rats were fasted overnight. The animals were sacrificed under ether anesthesia.

During the treatment period, rats were continuously monitored for behavioral (fearfulness, restlessness, alertness, irritability, and vomiting), neurological (gait, convulsion, touch/pain response, spontaneous activity, and bleeding orifices), and autonomic (defecation, micturition) changes. Animals were also observed for overall appearance and signs of toxicity such as postural abnormalities, convulsions or tremors, mydriasis or miosis, red deposits around eyes, nose, mouth, unusual respiration, vocalization, salivation, and head flicking, abnormal appearance of fur, compulsive biting, increased/decreased urination, circling and walking backward.

Fertility studies

Eight rats each from the Pb-exposed, diabetic, Pb-exposed diabetic, and control groups were individually placed in mating cages and were given 30 min adaptation period. A 90-day-old female that has been brought into pro-estrus (oestradiol benzoate 12 mg in olive oil injected subcutaneously 56 h prior to pairing plus progesterone 0.5 mg in olive oil injected subcutaneously 8 h prior to pairing) was placed in the cage for 7 days. The pre-coital sexual behavior (chasing, nosing, anogenital sniffing, genital grooming and attempted clasping and mounts) of the paired rats was observed 1-2 h later. Successful mating was confirmed by the presence of copulatory plugs and/or presence of sperm in the vaginal washings the following morning (06:00-07:00). The conception time, the interval between the first day of cohabitation and the day of plug/sperm, was recorded for each female. At day 15 post-coitum the mated females were subjected to laparotomy under ether anaesthesis. Both uteri were removed and examined for the number of conceptus (both live fetuses and implantations). In addition, both ovaries were removed and the number of corpora lutea was counted. The number of live embryos per dam, pre-implantation loss (difference between the number of corpora lutea and the number of implantations expressed as per number of corpora lutea), and post-implantation loss (difference between the number of implantation sites and the number of live fetuses expressed as per number of implantation sites) were determined.

Blood sugar levels

At the end of the experiment, all rats were fasted over night. Blood samples of rats were collected from the tail vein and glucose levels were determined using a semi-auto analyzer by glucose oxidase and peroxidase endpoint assay method using commercially available kits (Span diagnostics LTD, Surat, India).

Body and organ weights

The body weight of rats was recorded at the time of initiation and after completion of the experimental period (prior to necropsy). After completion of the experimental period, rats were fasted overnight, and killed by cervical dislocation. Whole blood was collected from the heart before necropsy. Serum was separated and stored at -20ºC until used. Brain, liver, kidney, spleen, testes, epididymis (caput, corpus, and cauda), vas deferens, seminal vesicles, prostate gland, and penis were dissected out, and attached tissue was removed and weighed to the nearest milligram on an electronic balance (Shimadzu Model No: BL- 220H; Kyoto, Japan). The tissue somatic index (TSI) was calculated by using the following formula:

TSI = (weight of the tissue in grams / weight of the body in grams) × 100.

Testes were used for determination of daily sperm production and biochemical analysis and cauda epididymides were used for sperm analysis. Caput, corpus, and cauda epididymis were used for biochemical studies.

Sperm parameters

Epididymal sperm analysis

Sperm were collected from right caudal (tail) part of the epididymis in 2.0 ml of M199 medium with 5 mg/mL fetal bovine serum. The sperm were counted by using Neubauer Chamber as described by Belsey *et al*.[19] The percentage of progressively motile sperm was evaluated microscopically within 5 min following their isolation from cauda epididymis at 37ºC.[19] The ratio of live to dead sperm was determined using 1% trypan blue reagent.[20]Sperm membrane integrity was assessed by exposing the sperm to hypo-osmotic solution and observed for tail swelling under the phase contrast microscope (HOS tail swelling). The percent of HOS tail swelled sperm was determined following the method described by Jeyendran *et al*.[21] The data

were expressed as millions/mL for sperm count and for other sperm parameters the data was expressed as percentage of total sperm.

Daily sperm production

Daily sperm production was determined in the testis by the method of Blazak *et al*.[22] Briefly, testis was decapsulated and homogenized in 50ml of ice-cold 0.9% NaCl solution containing 0.01% Triton X-100 using a sterilized mortar and pestle. The homogenate is allowed to settle for 1 minute and filtered through metal sieve, and the filtrate was used to count the homogenization-resistant spermatids using Neubauer haemocytometer. The number of sperm produced per gram tissue of testis per day was calculated.[23]

Assay of testicular steroidogenic marker enzymes

The testicular tissue was homogenized in ice-cold Tris-HCl buffer (pH 6.8). The activity levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) (EC 1.1.1.51) and 17β-hydroxysteroid dehydrogenases (17β-HSD) (EC 1.1.1.61) were determined in the testicular microsomal fractions by the method of Bergmeyer.[24] The enzyme assays were performed under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration. The reaction mixture in a volume of 2.0 ml contained 100 µmol sodium pyrophosphate buffer (pH 9.0) and 0.5 µmol cofactor NAD for 3β-HSD and NADPH for 17β-HSD, 0.08 µmol of substrate (dehydroepiandrosterone) for 3β-HSD and androstenedione for 17β-HSD) and 100 µl of enzyme source. The reactions were carried out in a quartz cuvette of 1.0 cm path length at 23±1ºC. The absorbance at 340 nm was measured at 20 sec intervals for 3 min in a Shimadzu UV-Visible spectrophotometer. The enzyme activities were expressed as nmol of NAD converted to NADH/mg protein/min (3β-HSD) or nmol of NADPH converted to NADP/mg protein/min (17β-HSD).

Determination of serum testosterone levels

The serum testosterone levels were determined by an ELISA method using a DRG testosterone ELISA kit (ELISA EIA-1559, 96 wells kit, DRG instrument, GmbH, Marburg, Germany). The assay was performed according to the user manual. The sensitivity, intra- and inter assay variation coefficients of kit were 0.083 ng/ml, 3.34 - 4.16 % and 4.73 - 9.94 % respectively.

Biochemical estimations in testis and different regions (caput, corpus and cauda) of epididymis

A 10% homogenate was prepared with glass-teflon homogenizer in ice-cold Tris buffer (2 mM, pH 7.4, contains 0.25 M sucrose) followed by centrifugation at 800 x *g* for 20 min at 4°C and clear supernatant was used for enzyme assays. LPx was estimated in terms of malondialdehyde (MDA) content and determined by using the thiobarbituric acid by the method of Ohkawa *et al*.[25] The LPx levels were expressed as µmol of MDA formed/g tissue. Superoxide anion production was analyzed based on the reduction of iodinitrotetrazolium violet using the method of Podczasy and Wei.[26] The levels of superoxide anion produced were expressed as nmoles/mg protein/min. Hydrogen peroxide (H_2O_2)generation was assessed by the method of Pick and Keisari.[27]The amount of H_2O_2produced was expressed as nmol H_2O_2 generated/mg protein/min. The reduced glutathione levels were estimated by using the procedure given by Ellman[28] and expressed as µmoles/g tissue. SOD activity was assayed by following the method described by Marklund and Marklund.[29] The SOD activity levels were expressed in nmoles pyrogallol oxidized/mg protein/min. Catalase (EC 1.11.1.6) was assayed by the method of Claiborne.[30] The activity of the enzyme was expressed in nmoles of H_2O_2 metabolized/mg protein/min. Glutathione peroxidase (EC 1.11.1.9) was assayed by the method of Paglia and

Valentine.[31] The activity was expressed as nmoles NADPH oxidized/mg protein/min. Glutathione reductase (EC 1.6.4.2) was assayed by the method of Carlberg and Mannervik.[32] The activity was expressed as nmoles NADPH oxidized/mg protein/min.

Determination of protein content

The protein concentration in the enzyme source was determined by using bovine serum albumin as the standard.[33]

Histological studies of the testis

Five- to seven millimeter-thick slices of tissue from the right testis were fixed in Bouin's fluid for 24h. Tissue samples were dehydrated in ascending alcohol series, cleared in xylene, embedded in paraffin wax, cut at 5 µm thickness, stained with hematoxylin, counter stained with eosin. Microscopic analysis for histological evaluation was made by using 40X objective and 10X ocular system of Olympus BX-41TF microscope. The area of testicular tissue components was determined measuring the area occupied by seminiferous tubules and interstitium. Twenty cross sections of stage VII-VIII seminiferous tubules from each animal were measured blindly for tubular diameter (basal lumina to basal lumina), epithelial height (basal lumina to neck of elongated spermatids), area of seminiferous tubule and inter-tubular space.

Statistical analysis of data

The statistical significance between control and treated groups was evaluated by one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) version 16.0 (SPSS Inc, Chicago, IL, USA) and the individual comparisons were obtained by Tukey's multiple range test. The data were expressed as mean $\pm$ standard deviation. Significance was set at $p< 0.05$.

Results

There were no significant differences in overall appearance, body position, activity, co-ordination or gait, lacrimation, vocalization, postural abnormalities in any of the control and experimental animals. Table 1 summarizes the effect of exposure to Pb on blood glucose levels and body weights of STZ induced diabetic rats. The mean blood glucose levels in control rats were 96.6 mg/dl. The mean blood glucose levels were significantly higher in the Pb-exposed (F = 716.52; df = 3, 20; p <0.001) and diabetic control group (F = 716.52; df = 3, 20; p <0.001) when compared to that of the control rats, whereas blood glucose levels of Pb exposed diabetic rats were comparable to that of the diabetic control group. There was no significant (p> 0.05) difference between the groups in initial body weights. The body weights of Pb-exposed rats did not show significant (p> 0.05) changes as compared with the control rats. Conversely, the body weights of diabetic control rats decreased significantly (F = 136.92; df = 3, 20; p <0.001) after the experimental period. Further, significant decrease in body weight (-9.64% and -47.21% compared to diabetic and Pb alone treated rats) was observed in Pb-exposed diabetic rats on the final day of the experiment.

The relative weights of brain, liver, kidney, spleen, corpus epididymis, vas deferens and penis were comparable (p> 0.05) in all groups (Table 2). Conversely, the weights of testes (F = 67.321; df = 3, 20; p <0.001), caput epididymis (F = 329.91; df = 3, 20; p <0.001), cauda epididymis (F = 30.571; df = 3, 20; p <0.001), seminal vesicles (F = 64.253; df = 3, 20; p <0.001) and prostate gland (F = 32.923; df = 3, 20; p <0.001) were significantly decreased in diabetic control and Pb-treated rats when compared to the control rats (Table 2). Further reduction in the relative weights of testis (-9.80% and -19.30% compared to diabetic and Pb alone treated rats), caput epididymis (-13.04% and -64.60% compared to diabetic and Pb alone treated rats), corpus epididymis (-33.33% and -42.86% compared to diabetic and Pb alone treated

rats), cauda epididymis (-33.33% and -50.00% compared to diabetic and Pb alone treated rats), seminal vesicles (-52.94% and -61.90% compared to diabetic and Pb alone treated rats), and prostate gland (-54.54% and -66.67% compared to diabetic and Pb alone treated rats) were observed in Pb-exposed diabetic rats (Table 2).

The total number of homogenization-resistant sperm per gram testis (F = 58.678; df = 3, 20; p <0.001), sperm numbers (F = 456.60; df = 3, 20; p <0.001), motile (F = 300.62; df = 3, 20; p <0.001), viable (F = 341.81; df = 3, 20; p <0.001) and HOS-tail swelled (F = 303.18; df = 3, 20; p <0.001) sperm in the epididymis were significantly decreased in the diabetic control and Pb-exposed rats, and comparatively, the decrease was much higher in daily sperm production (-36.32% and -49.36% compared to diabetic and Pb alone treated rats), sperm density (-79.09% and -84.09% compared to diabetic and Pb alone treated rats), motile (-84.72% and -87.21% compared to diabetic and Pb alone treated rats), viable (-83.74% and -87.42% compared to diabetic and Pb alone treated rats), and HOS-tail swelled (-80.42% and -83.69% compared to diabetic and Pb alone treated rats) sperm of Pb-exposed diabetic rats (Table 3).

The mean plasma concentration of testosterone was significantly (F = 97.769; df = 3, 20; p <0.001) lower in the diabetic control and Pb-exposed groups when compared with the control group. Additionally, further reduction in serum testosterone levels (-37.10% and -38.09% compared to diabetic and Pb alone treated rats) was observed in Pb-treated diabetic rats (Table 4). A significant decrease in the activity levels of 3β- (F = 70.232; df = 3, 20; p <0.001) and 17β-HSD (F = 49.029; df = 3, 20; p <0.001) was observed in the testis of rats exposed to Pb and diabetic control rats when compared to the controls. The decrease in the enzyme activities of 3β- (-23.03% and -40.89% compared to diabetic and Pb alone treated rats) and 17β-HSD (-22.92%

and -42.24% compared to diabetic and Pb alone treated rats) is much more pronounced in diabetic rats treated with Pb (Table 4).

The changes in antioxidant parameters in testis and different epididymal regions were presented in Tables 5-8. The levels of superoxide anion (F = 79.681 in testis, F = 73.338 in caput, F = 47.235 in corpus, and F = 57.610 in cauda epididymis; df = 3, 20; p <0.001*)*, H_2O_2 (F = 43.112 in testis, F = 43.181 in caput, F = 57.442 in corpus, and F = 52.727 in cauda epididymis; df = 3, 20; p <0.001*)*, and LPx (F = 45.262 in testis, F = 63.216 in caput, F = 48.581 in corpus, and F = 37.186 in cauda epididymis; df = 3, 20; p <0.001*)* were significantly elevated in testis, caput, corpus, and cauda epididymal parts of the diabetic control or Pb-treated rats when compared to that of the control rats. Further increase was observed in superoxide anion (26.78% and 64.55% in testis; 18.61% and 57.72% in caput epididymis; 24.90% and 53.88% in corpus epididymis; 23.27% and 44.07% in cauda epididymis compared to diabetic and Pb alone treated rats), H_2O_2 (21.87% and 42.71% in the testis; 34.49% and 68.77% in caput epididymis; 36.38% and 85.57% in corpus epididymis; 32.63% and 77.01% in cauda epididymis compared to diabetic and Pb alone treated rats)and LPx levels (34.98% and 63.12% in testis; 35.27% and 90.89% in caput epididymis; 37.96% and 106.74% in corpus epididymis; 75.64% and 102.13% in cauda epididymis compared to diabetic and Pb alone treated rats) in diabetic rats treated with Pb. Conversely, the levels of glutathione in testis (F = 47.801; df = 3, 20; p <0.001), caput (F = 45.393; df = 3, 20; p <0.001), corpus (F = 44.136; df = 3, 20; p <0.001), and cauda (F = 105.47; df = 3, 20; p <0.001) epididymal regions showed a significant decrease in diabetic control or Pb-exposed rat tissues when compared to the control rats. Pronounced decline in glutathione levels was observed in testis (-29.20% and -42.13% compared to diabetic and Pb alone treated rats), caput (-50.13% and -35.53% compared to diabetic and Pb alone treated rats), corpus (-30.17%

and -43.44% compared to diabetic and Pb alone treated rats), and cauda (-31.19% and -48.85% compared to diabetic and Pb alone treated rats) segments of Pb-exposed diabetic rats. The activities of antioxidant enzymes like superoxide dismutase (F = 43.020 for testis; F = 54.537 for caput epididymis; F = 38.869 for corpus epididymis; and F = 83.280 for cauda epididymis; df = 3, 20; p <0.001), catalase (F = 55.134 for testis; F = 20.892 for caput epididymis; F = 9.091 for corpus epididymis; and F = 17.798 for cauda epididymis; df = 3, 20; p <0.001), glutathione peroxidase (F = 55.117 for testis; F = 26.577 for caput epididymis; F = 32.177 for corpus epididymis; and F = 61.419 for cauda epididymis; df = 3, 20; p <0.001), glutathione reductase (F = 72.639 for testis; F = 53.493 for caput epididymis; F = 53.753 for corpus epididymis; and F = 47.473 for cauda epididymis; df = 3, 20; p <0.001), were significantly reduced in testis, caput, corpus, and cauda epididymis of diabetic control or Pb exposed rats. Furthermore, Pb-treated diabetic rats showed additional decrease in these enzyme activities of superoxide dismutase (-23.23% and -38.50% compared to diabetic and Pb alone treated rats in testis; -42.86% and -55.26% compared to diabetic and Pb alone treated rats in caput epididymis, -23.05% and -35.31% compared to diabetic and Pb alone treated rats in corpus epididymis; and -42.10% and -49.05% compared to diabetic and Pb alone treated rats in cauda epididymis), catalase (-17.66% and -36.25% compared to diabetic and Pb alone treated rats in testis; -27.34% and -33.00% compared to diabetic and Pb alone treated rats in caput epididymis, -12.44% and -25.05% compared to diabetic and Pb alone treated rats in corpus epididymis; and -9.40% and -14.78% compared to diabetic and Pb alone treated rats in cauda epididymis), glutathione peroxidase (-16.72% and -35.07% compared to diabetic and Pb alone treated rats in testis; -30.31% and -34.73% compared to diabetic and Pb alone treated rats in caput epididymis, -17.35% and -29.54% compared to diabetic and Pb alone treated rats in corpus epididymis; and -26.76% and -

40.05% compared to diabetic and Pb alone treated rats in cauda epididymis), glutathione reductase (-26.39% and -39.79% compared to diabetic and Pb alone treated rats in testis; -37.41% and -50.51% compared to diabetic and Pb alone treated rats in caput epididymis, -35.09% and -51.38% compared to diabetic and Pb alone treated rats in corpus epididymis; and -24.59% and -38.51% compared to diabetic and Pb alone treated rats in cauda epididymis) when compared to the diabetic control and Pb-exposed rats.

Histological examination of the testicular architecture of control rats displayed closely arranged seminiferous tubules with intact basement membrane followed by different stages of spermatogenic cells with fully mature sperms in the lumen (Fig 1A). Seminiferous tubules are round to elongate having large lumen with reduced spermatids (both round and elongated) and immature germ cells, and also with increased inter-tubular space in the diabetic rats (Fig 1B). In the Pb-exposed rats, although there were a few tubules in which immature germinal epithelial cells were observed in the lumen, the appearance was close to that of the control group (Fig 1C). Severely atrophied and irregularly placed small sized tubules with large interstitial space were observed in Pb-exposed diabetic rats (Fig. 1D). Further, disorganization in the seminiferous tubule basal membrane and epithelium, a decrease in germinal cells with loss of maturation, and unmatured germinal epithelial cells in the lumen were observed in Pb-exposed diabetic rats (Fig. 1D).

No significant ($p > 0.05$) reduction was observed in the seminiferous tubular diameter of diabetic rats when compared to that of control rats, whereas, a significant reduction was observed in the tubular diameter ($F = 133.67$; df = 3, 20; $p < 0.05$) of Pb-exposed rats. Epithelial height of diabetic ($F = 60.008$; df = 3, 20; $p < 0.05$) and Pb-exposed ($F = 60.008$; df = 3, 20; $p < 0.05$) rats was significantly reduced when compared to that of control rats (Table 9). Further, seminiferous

tubular area decreased significantly (F = 52.240; df = 3, 20; p< 0.05) with an increase in inter-tubular space in the testis of rats exposed to Pb (F = 106.56; df = 3, 20; p< 0.001) and diabetic control rats (F = 106.56; df = 3, 20; p< 0.01) when compared to the control rats. Significant reduction in the diameters of tubules (-42.90%; F = 133.67; df = 3, 20; p< 0.001 and -42.36%; F = 133.67; df = 3, 20; p< 0.001 compared to diabetic and Pb alone treated rats), thickness of germ cell layer (-48.66%; F = 60.008; df = 3, 20; p< 0.001 and -47.20%; F = 60.008; df = 3, 20; p< 0.001 compared to diabetic and Pb alone treated rats), tubular area (-34.28%;F = 52.240; df = 3, 20; p< 0.001 and -33.69%; F = 60.008; df = 3, 20; p< 0.001 compared to diabetic and Pb alone treated rats) and inter-tubular space (184.19%;F = 106.56; df = 3, 20; p< 0.001 and 171.23%; F = 106.56; df = 3, 20; p< 0.001 compared to diabetic and Pb alone treated rats) was observed after Pb-exposure in diabetic rats (Table 9).

All the fertility-related data were presented in Table 10. All the females mated with control and experimental males had copulatory plugs (mating index 100%), but only 4 out of 8 rats had implantations (fertility index = 50%) in Pb-exposed diabetic group. The mean number of corpora lutea was comparable in all groups. A significant increase was observed in the conception time (F = 88.866; df = 3, 24; p < 0.001) (interval between the day of cohabitation and the day of the presence of vaginal plug or the presence of sperm in vaginal smear) in females mated with diabetic males when compared to females mated with control males. The mean number of implantations (F = 4.336; df = 3, 24; p< 0.05) and mean number of live foetuses (F = 19.976; df = 3, 24; p < 0.001) were significantly decreased in females mated with Pb-treated diabetic males. Conversely, the mean pre- and post-implantation losses increased in females mated with diabetic or Pb-treated males. Further, a marked deterioration was also observed in all fertility related parameters (delay in conception time: 15.69% and 136.00% compared to diabetic

or Pb alone exposed rats; the mean number of implantations: -25.65% and -22.27% compared to diabetic or Pb alone exposed rats; the mean number of live foetuses: -56.10% and -52.55% compared to diabetic or Pb alone exposed rats) in females mated with diabetic males exposed to Pb.

Discussion

The present study was undertaken to assess the reproductive toxicity of Pb in STZ-induced diabetic rats. For this purpose, diabetes was induced in rats by a single intraperitoneal injection of STZ and maintained on lead (0.15%) containing water for 30 days and analyzed for spermatogenesis, steroidogenesis, antioxidant parameters in the testis and epididymis, histological and morphometric parameters in the testis and fertility parameters. The results of the present study show that Pb-induced reproductive toxicity is exacerbated in diabetic rats.

The present work has shown that exposure of rats to Pb to a concentration of 0.15% through drinking water did not affect body weight gains compared to control rats suggesting that exposure to Pb did not impose undue stress on the rats. Conversely, the body weights of diabetic control and diabetic Pb-exposed rats were significantly reduced, probably due to excessive utilization of protein.[34] The results also showed that treatment of rats with STZ and/or Pb caused no significant change in the relative weights of brain, liver, kidney and spleen indicating normal metabolic condition of the animals. Additionally, there was a significant reduction in the weights of testes, epididymis, seminal vesicles and prostate gland among experimental rats compared to control rats, an observation similar to that made earlier for diabetic[16] and Pb-exposed rats[10] suggesting testicular and epididymal toxicity. Moreover, a significant decline in the weights of the reproductive tissues may be due to low serum testosterone level, since testosterone plays a major role in regulating the structural integrity and functional activities of testes and accessory

sex organs.[35] In the present study, the serum testosterone levels decreased significantly in diabetic and/or Pb-treated rats, designating that reproductive tissues in experimental rats are more prone to toxic insults than non-reproductive tissues (brain, liver, kidney and spleen).

The present work has also shown that testicular daily sperm production, epididymal sperm concentration, motile, viable and HOS-tail swelled sperms were significantly decreased in both diabetic control and Pb-treated rats. These results are in par with earlier reports.[36,37] Reduction in epididymal sperm concentration, motile, viable and HOS-tail swelled sperms may be due to the combined effect of decreased Leydig cell function and elevated oxidative toxicity.[4] A decline in Leydig cell function can be evidenced by low testosterone levels in the serum/testis. Sperm being rich in unsaturated fatty acids exhibit high instability to elevated oxidative stress. In addition, damage from oxidative stress by free radicals deteriorates the sperm motility and viability. The detection of high levels of MDA is negatively correlated with the motility parameters and ability of the spermatozoa to penetrate the zona pellucida.[38]

The data also reveal reduced levels of serum testosterone in experimental rats, indicating a probable inhibition of androgen synthesis. Thus the decrease in circulatory testosterone levels could be due to diminished responsiveness of Leydig cells to LH and/or direct inhibition of testosterone synthesis in experimental rats. Furthermore, there was a significant decrease in the activity levels of 3β- and 17β-HSDs in the testis of experimental rats. Our results are also in accordance with earlier reports of diabetic[39,40] and Pb treated rats.[13] The 3β- and 17β-HSDs are the vital enzymes in the biosynthetic pathway of testosterone. The reduced activities of these enzymes may results in reduced steroidogenesis in rat. The diminution in the activities of these enzymes in testis and decreased serum testosterone levels may also be due to elevated oxidative stress.[9,13]

Sperm production and maturation are very sensitive to pro-oxidant/antioxidant balance. Low levels of ROS are essential for hyper-activation motility, capacitation/acrosome reaction and other physiological processes.[41] However, spermatozoa are particularly susceptible to oxidative injury due to the abundance of plasma membrane polyunsaturated fatty acids.[42] These unsaturated fatty acids provide fluidity that is necessary for membrane fusion events (e.g., the acrosome reaction and sperm-egg interaction)and for sperm motility. However, the unsaturated nature of these molecules predisposes them to free radical attack and ongoing LPx throughout the sperm plasma membrane. This phenomenon has deleterious effects on the fluidity, integrity and flexibility of sperm plasma membrane, characteristics associated with fertilizing capacity. Once this process has been initiated, accumulation of lipid peroxides occurs on the sperm surface (resulting in loss of sperm motility) and oxidative damage to DNA can ensue.[43] In the present study, activity levels of SOD, catalase, glutathione peroxidase and glutathione reductase decreased significantly with an elevation of superoxide, H_2O_2levels and LPx products in the testes and different parts of epididymis of experimental rats. Further, we observed a significant decline in testicular and epididymal glutathione levels in experimental rats. Glutathione (GSH) is the most abundant non-enzymatic antioxidant which is crucial for protecting the cells from oxidative stress by its free radical scavenging activity and inhibitory action on LPx.[44]

SOD is the primary superoxide scavenger catalyzes the dismutation of superoxide radicals into H_2O_2, which is then detoxified by catalase into water and oxygen. Both these enzymes cooperate together; catalase decomposes hydrogen peroxide formed during the SOD-catalyzed dismutation reaction of superoxide radical anion. Previously, it is reported that a decrease in the activity levels of SOD caused an increase in the levels of superoxide anion radicals, which inactivates catalase activity.[45] The reduction in the activity levels of catalase fails

to eliminate H_2O_2 from the cell; the accumulated H_2O_2 is known to inactivate SOD.[46] As H_2O_2 is lipophilic in nature it can traverse through the plasma membrane and affects cellular constituents thereby results in oxidative stress by decreasing the enzymatic defense system. Previously, it is reported that diabetic condition or exposure to Pb can stimulate oxidative stress by triggering the generation of ROS and by reducing the antioxidant defense system.[6,40,47]

Another remarkable effect observed in the present study is a decrease in the activities of glutathione-related enzymes. Glutathione peroxidase catalyses the reduction of lipid peroxides and hydrogen peroxide at the expense of glutathione.[48] Reduced glutathione peroxidase activity causes accumulation of lipid peroxides and other oxidants which make the cellular membranes more susceptible to the oxidative damage. The activity of glutathione peroxidase is coupled to glutathione reductase, which regenerates reduced form of glutathione from glutathione disulphide (GSSG).[49] Our data show that the activity of glutathione reductase and glutathione levels also significantly decreased in the tissues of experimental rats. Hence, the observed reduction in the activity of glutathione peroxidase activity might be due to depletion of glutathione in the experimental rat tissues.

Consistent with the results of sperm parameters and biochemical changes, alterations in testicular architecture were observed in Pb-exposed and diabetic control males, including loss of compactness, disorganized spermatogenesis, lumen with immature germinal cells, in addition to enlarged inter-tubular spaces. This shows that spermatogenesis was not completed and was impaired. This may be associated with a lack of insulin, which down regulates the insulin mediated stimulation of androgen biosynthesis and cell proliferation in the testis of diabetic control rats and reduced steroidogenesis in Pb-exposed rats. Furthermore, severe histopathological changes observed in Pb-exposed diabetic rats might be due to synergistic effect

of both Pb and diabetis in further reducing androgen synthesis. These alterations may also be due to elevated oxidative toxicity in the testis of experimental rats. Intensification of oxidative stress in experimental rats may disrupt the integrity of mitochondrial and acrosomal membranes and thereby affects motility and fertilizing capacity of sperm.

The final reproductive endpoint that was tested was the male's ability to sire offspring. Of the females that were cohabited with control, Pb-exposed and diabetic rats all had copulatory plugs, clearly suggesting that induction of diabetes or exposure to Pb at a concentration of 0.15% did not affect male fertility; whereas only 50% rats cohabited with Pb-exposed diabetic males had copulatory plugs. Whether reduced fertility observed in Pb-exposed diabetic rats resulted from lower sperm numbers, altered motility, or depressed sexual desire cannot be determined from the present fertility data. However, the absence of copulatory plugs in four of eight females may indicate depressed sexual behavior in Pb-exposed diabetic males. Additional observations of fewer embryos per dam and higher pre-implantation loss in Pb-exposed diabetic rats suggest compromised sperm fertility. Hence, further in-depth fertility studies are warranted involving observations on mount frequency, intromission frequency, mount latency, intromission latency, and ejaculatory latency. In addition, artificial insemination using a fixed number of sperm from the cauda of epididymis will be an ideal method for comparing sperm fertility between the control and experimental animals.

Finally, the effects that were observed for several end points in either diabetic control or Pb exposed rats were more pronounced in diabetic rats exposed to Pb. Exposure to Pb has been found to exacerbate diabetes-induced reproductive toxicity including reduction in spermatogenesis, steroidogenesis and suppression in fertility and an elevation in oxidative toxicity as compared to diabetic control or Pb alone treated rats. While serving our purpose of

providing reference end points, the stresses selected in the present study clearly do not represent all environmental stresses experienced by humans and wildlife. The nature of interactions between such stresses may be plausible in the general environment.

In conclusion, the current study presents a possible pathway of sexual dysfunction in diabetic and Pb-exposed rats. First, these stresses induce oxidative toxicity in testis and epididymis by decreasing anti-oxidant enzyme activities leading to elevated levels of ROS. Subsequently, testicular steroidogenesis is reduced in diabetic control and Pb-exposed rats. Consequently, spermatogenesis is reduced in response to reduced steroidogenesis and elevated ROS. However, at the present time, the exact contribution of oxidative toxicity versus compromised steroidogenesis resulting in jeopardized sperm quality and quantity cannot be determined. On the other hand, when we expose diabetic rats to Pb, they showed an increase in the reproductive toxic responses observed in diabetic control or Pb-treated rats. Indeed, the enhanced reduction in steroidogenesis and elevation in oxidative toxicity observed in diabetic rats exposed to Pb could be relevant to explain the basis for the further reduction in sperm production and deterioration in sperm quality thereby affecting fertility. Extrapolation of rat data to human being is always difficult. Regardless, our study demonstrates that diabetic individuals have to be cognizant of their unfavorable reproductive risk profile.

Guidelines for ethical approval

Authors declare that the experiments were consistent with the guidelines and principles of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India and approved by the Institutional Animal Ethical Committee at S.V. University, Tirupati, India (vide No. IAEC/No- 438/01/a/CPCSEA) with a resolution No: 57/2012/(i)/a/CPCSEA/IAEC/SVU/PSR- KPR dt.08-07-2012.

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Conflict of Interest

The author(s) declare that no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Legend for figure

Fig. 1. Photomicrographs of H&E-stained sections of testes. Scale bar = 50µm.

A) Testicular sections of control rat showing well organized seminiferous tubules with normal spermatogenesis (→).

B) Testicular sections of diabetic control rat showing immature germ cells in the lumen (Δ)with enlarged inter-tubular spaces(*)

C) Testicular sections of Pb-exposed rat showing immature germ cells in the lumen (Δ)and enlarged inter-tubular spaces (*)

D) Testicular sections of Pb-exposed diabetic rat showing the smaller seminiferous tubules with a remarkable increase in the inter-tubular space (*) and disorganized, irregular, and thin basement membrane.

Table 1. Effect of Pb on blood glucose levels and body weight (g) of STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
Glucose (mg/dl)	$96.60^{a} \pm 7.42$	$323.65^{b} \pm 9.78$ (235.04)	$209.34^{c} \pm 11.23$ (116.71)	$339.65^{b} \pm 12.33$ (251.60)
Initial body weight (g)	$226.33^{a} \pm 10.33$	$231.50^{a} \pm 6.09$ (2.28)	$232.83^{a} \pm 8.52$ (2.87)	$229.50^{a} \pm 7.61$ (1.40)
Final body weight (g)	$299.33^{a} \pm 2.58$	$172.83^{b} \pm 6.24$ (-42.26)	$295.83^{a} \pm 4.71$ (-1.17)	$156.17^{c} \pm 3.76$ (-47.83)
Weight gain/loss (g)	$73.00^{a} \pm 9.25$	$-58.67^{b} \pm 7.71$ (-180.37)	$63.00^{a} \pm 7.48$ (-13.70)	$-73.33^{c} \pm 9.29$ (-200.45)

Values are mean ± S.D. of 6 individuals.
Values in the parenthesis are percent change from control
Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 2. Effect of Pb on tissue somatic index (W/W%) in STZ-induced diabetic rats

Tissue	Control	Diabetic	Pb	Diabetic + Pb
Brain	0.64^{a}±0.05	0.66^{a} ± 0.03 (3.12)	0.59^{a} ± 0.03 (-7.81)	0.67^{a} ± 0.03 (4.69)
Liver	2.87^{a} ± 0.20	2.25^{b} ± 0.34 (-21.60)	2.83^{a} ± 0.29 (-1.39)	3.28^{a} ± 0.36 (14.28)
Kidney	0.54^{a}± 0.03	0.53^{a}± 0.03 (-1.85)	0.46^{a}± 0.08 (-14.81)	0.60^{a}± 0.06 (12.96)
Spleen	0.37^{a}± 0.03	0.30^{a}± 0.08 (-18.92)	0.40^{a}± 0.03 (8.11)	0.34^{a}± 0.07 (-8.11)
Testis	0.74^{a}± 0.06	0.51^{b}± 0.02 (-31.08)	0.57^{c}± 0.02 (-22.97)	0.46^{d}± 0.03 (-37.84)
Caput epididymis	1.21^{a} ± 0.08	0.46^{b} ± 0.03 (-61.98)	1.13^{a} ± 0.05 (-6.61)	0.40^{b} ± 0.06 (-66.94)
Corpus epididymis	0.07^{a} ± 0.01	0.06^{ab} ± 0.01 (-14.28)	0.07^{a} ± 0.01 (0)	0.04^{b}± 0.02 (-42.86)
Cauda epididymis	0.11^{a} ± 0.02	0.06^{b} ± 0.01 (-45.45)	0.08^{b} ± 0.01 (-27.27)	0.04^{c} ± 0.01 (-63.64)
Vas deferens	0.10^{a} ± 0.01	0.06^{b} ± 0.01 (-40.00)	0.11^{a} ± 0.02 (10.00)	0.08^{ab} ± 0.02 (-20.00)
Seminal Vesicles	0.54^{a} ± 0.06	0.34^{b} ± 0.03 (-37.04)	0.42^{c} ± 0.05 (-22.22)	0.16^{d} ± 0.05 (-70.37)
Prostate	0.19^{a} ± 0.03	0.11^{b} ± 0.03 (-42.10)	0.15^{ab} ± 0.02 (-21.05)	0.05^{c} ± 0.02 (-73.68)
Penis	0.11^{a} ± 0.02	0.14^{a} ± 0.03 (27.27)	0.11^{a} ± 0.02 (0)	0.15^{a} ± 0.02 (45.45)

Values are mean ± S.D. of 6 individuals

Values in the parenthesis are percent change from control

Mean values with different superscripts in a row differ significantly from each other at $p< 0.05$

Table 3. Effect of Pb on daily sperm production and epididymal sperm parameters in STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
DSP (millions/g testis)	22.73^{a} ±2.70	14.84^{b} ±1.71 (-34.71)	18.66^{c} ±1.25 (-17.90)	9.45^{d} ±1.14 (-58.42)
Sperm Count (millions/mL)	71.13^{a} ±3.76	41.33^{b} ±3.30 (-41.89)	54.3^{c} ±3.13 (-23.66)	8.64^{d}± 1.40 (-87.85)
Motile sperm (%)	73.50^{a} ±4.76	44.5^{b} ±3.02 (-39.45)	53.17^{c} ±4.87 (-27.66)	6.80^{d}± 2.59 (-90.75)
Viable sperm (%)	74.83^{a} ±4.71	45.5^{b} ±3.27 (-39.19)	58.83^{c} ±4.12 (-21.38)	7.40^{d} ± 2.88 (-90.11)
HOS-tail swelled sperm (%)	67.67^{a} ±3.56	39.83^{b} ±4.40 (-41.14)	47.83^{c} ±3.66 (-29.32)	7.80^{d} ± 1.92 (-88.47)

Values are mean ± S.D. of 6 individuals

Values in the parenthesis are percent change from control

Mean values with different superscripts in a row differ significantly from each other at $p< 0.05$

Table 4. Effect of Pb on testicular 3 β-HSD and 17 β-HSD activity levels and serum testosterone levels in STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
3β-HSD (n moles of NAD converted to NADH/mg protein/min)	$20.29^{a} \pm 1.08$	$12.81^{b} \pm 1.43$ (-36.86)	$16.68^{c} \pm 1.67$ (-17.79)	$9.86^{d} \pm 1.03$ (-51.40)
173β-HSD (n moles of NADPH converted to NADP/mg protein/min	$14.80^{a} \pm 1.29$	$8.64^{b} \pm 0.77$ (-41.62)	$11.53^{c} \pm 1.92$ (-22.09)	$6.66^{d} \pm 0.46$ (-55.00)
Testosterone (ng/mL)	$2.85^{a} \pm 0.19$	$1.86^{b} \pm 0.18$ (-34.74)	$1.89^{b} \pm 0.17$ (-33.68)	$1.17^{c} \pm 0.14$ (-58.95)

Values are mean ± S.D. of 6 individuals

Values in the parenthesis are percent change from control

Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 5. Effect of Pb on antioxidant parameters in testis of STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
Superoxide anion (n moles/mg protein/min)	9.25^{a}± 0.96	14.90^{b}± 1.36 (61.08)	11.48^{c}± 1.08 (24.11)	18.89^{d}± 1.18 (104.22)
Hydrogen peroxide (n moles/mg protein/min)	19.06^{a} ± 3.01	27.39^{b} ± 1.84 (43.70)	23.39^{c} ± 1.26 (22.72)	33.38^{d} ± 2.57 (75.13)
Lipid peroxidation (µ moles of malondialdehyde/g tissue)	5.62^{a} ± 0.55	8.29^{b} ± 0.84 (47.51)	6.86^{c} ± 0.61 (22.06)	11.19^{d} ± 1.29 (99.11)
Superoxide dismutase (n moles pyrogallol oxidized/mg protein/min)	6.77^{a} ± 0.73	4.39^{b} ± 0.26 (-35.15)	5.48^{c} ± 0.59 (-19.05)	3.37^{d} ± 0.49 (-50.22)
Catalase (n moles of H_2O_2 metabolized/mg protein/min)	17.98^{a} ± 1.42	11.04^{b} ± 1.34 (-38.60)	14.26^{c} ± 1.46 (-20.69)	9.09^{b} ± 0.81 (-49.44)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	48.30^{a} ± 5.13	29.54^{b} ± 1.75 (-38.84)	37.89^{c} ± 3.97 (-21.55)	24.60^{d} ± 1.33 (-49.07)
Glutathione reductase (n moles NADPH oxidized/mg protein/min)	46.14^{a} ± 2.37	31.90^{b} ± 4.17 (-30.86)	39.00^{b} ± 2.53 (-15.47)	23.48^{c} ± 1.30 (-49.11)
Reduced glutathione (µ moles/g tissue)	57.92^{a} ± 6.55	37.22^{b} ± 3.21 (-35.74)	45.53^{c} ± 4.93 (-21.39)	26.35^{d} ± 3.43 (-54.51)

Values are mean ± S.D. of 6 individuals

Values in the parenthesis are percent change from control

Mean values with different superscripts in a row differ significantly from each other at $p< 0.05$

Table 6. Effect of Pb on antioxidant parameters in caput epididymis of STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
Superoxide anion (n moles/mg protein/min)	$7.62^{a} \pm 0.56$	$13.43^{b} \pm 1.12$ (76.25)	$10.10^{c} \pm 1.32$ (32.54)	$15.93^{d} \pm 1.03$ (109.05)
Hydrogen peroxide (n moles/mg protein/min)	$11.52^{a} \pm 1.91$	$17.92^{b} \pm 1.8$ (55.55)	$14.28^{c} \pm 0.60$ (13.89)	$24.10^{d} \pm 3.03$ (109.20)
Lipid peroxidation (μ moles of malondialdehyde/g tissue)	$3.55^{a} \pm 0.36$	$6.35^{b} \pm 0.46$ (78.87)	$4.50^{c} \pm 0.53$ (26.76)	$8.59^{d} \pm 1.12$ (141.97)
Superoxide dismutase (n moles pyrogallol oxidized/mg protein/min)	$5.37^{a} \pm 0.56$	$3.57^{b} \pm 0.28$ (-33.52)	$4.56^{c} \pm 0.69$ (-15.08)	$2.04^{d} \pm 0.19$ (-62.01)
Catalase (n moles of H_2O_2 metabolized/mg protein/min)	$9.10^{a} \pm 1.14$	$6.40^{b} \pm 1.08$ (-29.67)	$6.94^{b} \pm 1.03$ (-23.74)	$4.65^{c} \pm 0.58$ (-42.97)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	$24.19^{a} \pm 3.09$	$17.88^{b} \pm 0.89$ (-26.08)	$19.09^{ab} \pm 2.39$ (-21.08)	$12.46^{c} \pm 2.21$ (-48.49)
Glutathione reductase (n moles NADPH oxidized/mg protein/min)	$21.80^{a} \pm 1.57$	$14.62^{b} \pm 1.39$ (-32.93)	$18.49^{c} \pm 2.08$ (-15.18)	$9.15^{d} \pm 2.13$ (-58.03)
Reduced glutathione (μ moles/g tissue)	$22.10^{a} \pm 1.81$	$18.45^{b} \pm 1.50$ (-16.51)	$14.27^{c} \pm 2.47$ (-35.43)	$9.20^{d} \pm 2.16$ (-58.37)

Values are mean ± S.D. of 6 individuals

Values in the parenthesis are percent change from control

Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 7. Effect of Pb on antioxidant parameters in corpus epididymis of STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
Superoxide anion (n moles/mg protein/min)	8.58^{a}± 0.58	13.01^{b}± 1.50 (51.63)	10.56^{c}± 0.90 (23.08)	16.25^{d}± 1.47 (89.39)
Hydrogen peroxide (n moles/mg protein/min)	6.05^{a} ± 1.81	12.45^{b} ± 1.2 (105.78)	9.15^{b} ± 1.38 (51.24)	16.98^{c} ± 1.59 (180.66)
Lipid peroxidation (µ moles of malondialdehyde/g tissue)	2.46^{a} ± 0.44	5.11^{b} ± 0.63 (107.72)	3.41^{c} ± 0.41 (38.62)	7.05^{d} ± 1.12 (186.58)
Superoxide dismutase(n moles pyrogallol oxidized/mg protein/min)	3.74^{a} ± 0.34	2.69^{b} ± 0.36 (-28.07)	3.20^{c} ± 0.17 (-14.44)	2.07^{d} ± 0.20 (-44.65)
Catalase (n moles of H_2O_2 metabolized/mg protein/min)	6.2^{a} ± 0.49	4.34^{b} ± 1.04 (-30.00)	5.07^{b} ± 0.56 (-18.22)	3.80^{b} ± 1.10 (-38.71)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	32.13^{a} ± 3.75	21.33^{b} ± 2.46 (-33.61)	25.02^{b} ± 2.84 (-22.13)	17.63^{c} ± 0.61 (-45.13)
Glutathione reductase (n moles NADPH oxidized/mg protein/min)	29.11^{a} ± 2.41	17.41^{b} ± 2.97 (-40.19)	23.24^{c} ± 2.13 (-20.16)	11.3^{d} ± 2.64 (-61.18)
Reduced glutathione (µ moles/g tissue)	31.35^{a} ± 3.55	21.68^{b} ± 2.83 (-30.84)	26.77^{c} ± 1.05 (-14.61)	15.14^{d} ± 2.15 (-51.71)

Values are mean ± S.D. of 6 individuals

Values in the parenthesis are percent change from control

Mean values with different superscripts in a row differ significantly from each other at $p< 0.05$

Table 8. Effect of Pb on antioxidant parameters in cauda epididymis of STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
Superoxide anion (n moles/mg protein/min)	8.90^{a}± 1.05	13.02^{b}± 0.82 (46.29)	11.14^{c}± 0.76 (25.17)	16.05^{d}± 1.21 (80.34)
Hydrogen peroxide (n moles/mg protein/min)	17.85^{a} ± 0.98	28.44^{b} ± 2.71 (37.24)	21.31^{c} ± 1.41 (19.38)	37.72^{d} ± 4.98 (111.32)
Lipid peroxidation (µmoles of malondialdehyde/g tissue)	4.99^{a} ± 0.72	7.02^{b} ± 0.83 (40.68)	6.10^{c} ± 0.39 (22.24)	12.33^{d} ± 2.34 (147.09)
Superoxide dismutase (n moles pyrogallol oxidized/mg protein/min)	4.59^{a} ± 0.36	3.23^{b} ± 0.20 (-29.63)	3.67^{b} ± 0.29 (-20.04)	1.87^{c} ± 0.34 (-59.26)
Catalase (n moles of H_2O_2 metabolized/mg protein/min)	12.97^{a} ±1.38	9.04^{b} ± 0.79 (-30.30)	9.61^{b} ± 1.75 (-25.90)	8.19^{b} ± 0.57 (-36.85)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	42.63^{a} ± 3.6	27.8^{b} ±2.25 (-34.79)	33.96^{c} ± 3.79 (-20.34)	20.36^{d} ± 1.56 (-52.24)
Glutathione reductase (n moles NADPH oxidized /mg protein/min)	39.96^{a} ± 2.25	27.00^{b} ± 3.59 (-32.43)	33.11^{b} ± 3.89 (-17.14)	20.36^{c} ± 1.56 (-49.05)
Reduced glutathione (µ moles/g tissue)	44.91^{a}± 2.86	28.15^{b} ± 2.39 (-37.32)	37.87^{c} ± 2.87 (-15.67)	19.37^{d} ± 2.5 (-56.87)

Values are mean ± S.D. of 6 individuals.

Values in parenthesis are percent change from control

Mean values with different superscripts in a row differ significantly from each other at $p< 0.05$

Table 9. Effect of Pb on testicular morphometric parameters of STZ-induced diabetic rats

Parameter	Control	Diabetic	Pb	Diabetic + Pb
Seminiferous tubular diameter (μm)	335.42[a] ±16.53	312.49[a]±13.28 (-6.83)	309.56[b]±18.36 (-7.71)	178.44[c]±11.23 (-46.80)
Seminiferous epithelium height (μm)	123.58[a] ±11.24	108.43[b] ±10.13 (-12.26)	105.44[b]±8.23 (-14.68)	55.67[c]±7.16 (-54.95)
Area of seminiferous tubule (%)	94.87[a] ±5.65	84.31[b] ±5.89 (-11.13)	83.56[b]±4.93 (-11.92)	55.41[c]±6.34 (-41.59)
Inter-tubular space (%)	5.13[a] ±0.41	15.69[b] ±2.13 (205.85)	16.44[b] ±2.98 (220.47)	44.59[c] ±7.12 (769.2)

Values are mean ± S.D. of 6 individuals.
Values in the parentheses are percent change from that of control.
Mean values with same superscript do not differ significantly from each other. $p< 0.05$.

Table 10. Effect of Pb on fertility of STZ-induced diabetic rats

Parameter	Control[#]	Diabetic[#]	Pb[#]	Diabetic + Pb[$]
Pregnant/mated	8/8	8/8	8/8	4/8
Conception time (days)	2.3[a] ±0.41	5.1[b] ±0.56 (121.74)	2.5 [a] ±0.32 (8.69)	5.9[b] ±0.71 (156.52)
Number of corpora lutea/dam	13.5[a] ±1.22	13.9[a] ±1.14 (2.96)	13.3[a] ±1.21 (-1.48)	13.8[a] ±1.33 (2.22)
Number of implantations/dam	12.8[a] ±1.12	11.5[a] ±1.82 (-10.16)	11.0[a] ±1.76 (-14.06)	8.55[b] ±1.12 (-33.20)
Pre-implantation loss (%)	5.19	7.27	17.29	38.04
Number of live fetuses/dam	12.4[a] ±1.11	9.75[a] ±1.32 (-21.37)	9.02[b] ±1.87 (-27.26)	4.28[c] ±1.83 (-65.48)
Post-implantation loss (%)	3.13	15.22	18.0	49.94

Values are mean ± S.D. [#]n=8; [$]n=4

Values in the parentheses are percent change from that of control.

Mean values with same superscript do not differ significantly from each other. $p< 0.05$.

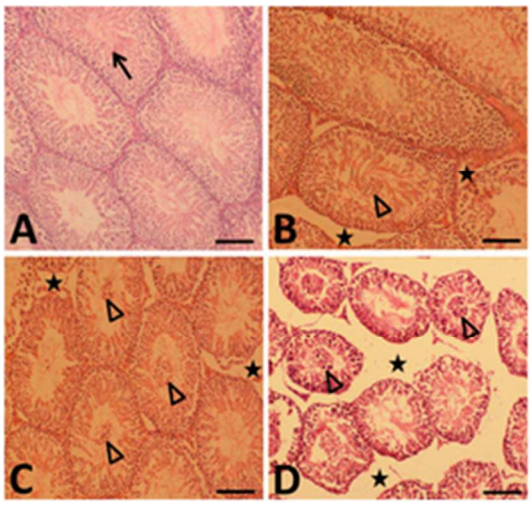

Fig. 1. Photomicrographs of H&E-stained sections of testes. Scale bar = 50μm.

A) Testicular sections of control rat showing well organized seminiferous tubules with normal spermatogenesis (→).
B) Testicular sections of diabetic control rat showing immature germ cells in the lumen (Δ) with enlarged inter-tubular spaces (*)
C) Testicular sections of Pb-exposed rat showing immature germ cells in the lumen (Δ) and enlarged inter-tubular spaces (*)
D) Testicular sections of Pb-exposed diabetic rat showing the smaller seminiferous tubules with a remarkable increase in the inter-tubular space (*) and disorganized, irregular, and thin basement membrane.

11x10mm (600 x 600 DPI)