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Acute diethyl nitrosamine and cadmium co-exposure exacerbates deficits in endocrine balance, sperm characteristics and antioxidant defence mechanisms in testes of pubertal rats

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Abstract

Diethylnitrosamine (DEN) and cadmium are environmental contaminants of known poisonous consequences in animals and humans. We examined the influence of acute oral co-exposure to DEN (10 mg/kg) and cadmium (5 mg/kg) on endocrine balance, semen and antioxidant status in rat testes. The results indicated decreases ($p < 0.05$) in the weight of the testis and organo-somatic index of the testes in rats administered with either DEN or cadmium were aggravated in the co-exposed rats. Serum concentrations of follicle-stimulating hormone (FSH), luteinising hormone (LH) and testosterone decreased, and were more pronounced in rats co-treated with DEN and cadmium. Enzymatic and non enzymatic antioxidant activities decreased following separate exposure to DEN and cadmium, and were increased in rats co-treated with DEN and cadmium. The significant ($p < 0.05$) increases in malondialdehyde (MDA) was complemented by marked increase in sperm abnormalities, reduction in the sperm count, motility and viability compared with control. Histologically, co-exposure to DEN and cadmium aggravates their discrete effects on the testes. Co-exposure to DEN and cadmium elicited more severe endocrine disruption and testicular oxidative damage in rats, revealing additive adverse effects on testicular functions in rats and as such, may put exposed individual at greater risk.

KEYWORDS

cadmium, co-exposure, Diethylnitrosamine, rats, testicular dysfunction

1 | INTRODUCTION

The various adverse health effects posed by exposure to environmental toxicants are of great concern to the general public including policymakers, medical practitioners and scholars globally. Cadmium is a well-known ubiquitous transition metal with great harmful effects in living organisms. Cadmium is essential in the production of batteries, plastics and alloys. The general public is exposed to cadmium via inhalation of polluted dust, ingestion of contaminated agricultural produce, food and cigarette smoking (Chavez et al., 2015; Gaur & Agnihotri, 2018). Cadmium upon absorption has been reported to bioaccumulates in the renal, hepatic and reproductive tissues (Klaassen, Liu, & Diwan, 2009). Previous investigations have demonstrated that testes is particularly vulnerable to the action of xenobiotics including metals owing to the several and continuous divisions that occur in germinal cells during maturation and differentiation processes (Bonde, 2010).Cadmium exposure reportedly disrupted endocrine function, induced oxidative stress and apoptosis as well as decreased sperm function in animals and humans (Siu, Mruk, Porto, & Cheng, 2009; Takiguchi & Yoshihara, 2006).

Diethylnitrosamine (DEN) is an archetypal hepatocarcinogen widely present in various foods, alcohol, bacon, smoked meats and

tobacco smoke (Herrmann, Duedahl-Olesen, Christensen, Olesen, & Granby, 2015). The principal source of N-nitrosamines is protein often generated from nitrate precursor. Moreover, the intestinal gastric acid has been demonstrated to activate amines to N-nitrosamines (Sierra et al., 1991). Generally, the hepatic cytochrome P-450 system is well known to metabolise N-nitrosamines to alkylating agents leading to induction of reactive oxygen species (ROS) and oxidative stress (Sheweita, 2000). Further, the cytochrome P-450 isoenzymes are xenobiotic metabolising enzymes located in the mitochondrial membrane or the endoplasmic reticulum where they play a pivotal role in steroidogenesis. DEN can pass through the blood–testis barrier and consequently be metabolised in the spermatogonia to an alkylating derivative. Besides, externally activated DEN metabolites can pass through the barrier (Kamino et al., 1995).

The pubertal stage is an important phase because the developing organism is commonly more susceptible to toxic insult than the adult. Up till now, limited report is available in the scientific literature on the effect of pubertal co-exposure to cadmium and DEN on the male reproductive system. Given the possibility of dietary co-exposure to these contaminants, the purpose of the present study was to investigate whether simultaneous exposure to cadmium and DEN would result in antagonistic or synergistic effects in testes of pubertal rats.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Diethylnitrosamine (DEN ≥99%), cadmium chloride (CdCl2; 99.99%), thiobarbituric acid, (TBA) glutathione (GSH), hydrogen peroxide, 5', 5'-dithio-bis-2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB) and adrenaline acquired from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 | Experimental animal care

Forty Wistar rats (male) approximately 5 weeks of age and average weight of 110 ± 2 g purchased from the animal breeding facility, Faculty of Veterinary Medicine, University of Ibadan, Nigeria, were used for this study. The rats were housed in rodent cage situated in the Department of Biochemistry and kept under conditions of 12-hr light/dark cycle. All animals had free access to rat pellets and water. Experimental animals were acclimatised for seven days before commencement of the study and were humanely treated in adherence to the conditions required for the Care and Use of laboratory experimental animals as stipulated by the National Institute of Health (NIH). All procedures were done following authorisation by the University of Ibadan Ethical Committee.

2.3 | Study design

Four groups each of (n = 10 rats) were treated with DEN and cadmium for seven consecutive days as follows: 1: Control animals received 2 ml/kg of corn oil; 2: DEN (10 mg/kg dissolved in corn oil); 3: $CdCl_2$ (5 mg/kg dissolved in water); and 4: DEN (10 mg/kg) and $CdCl_2$ (5 mg/kg).The doses of DEN and $CdCl_2$ were chosen from earlier published studies (Arafa, Mohammad, & Atteia, 2014; Ujah et al., 2018; Zhang et al., 2012) and administered per oral.

2.4 | Animal sacrifice

The study was terminated 24 hr following the last treatment, animals were weighed, blood sample collected (via retro-orbital puncture) and thereafter sacrificed by cervical dislocation. Blood serum was obtained by centrifugation (3,000 *g* for 10 min) and stored (−20°C) until required for the assessment of reproductive hormones levels by ELISA kits. The testes were removed immediately, weighed and thereafter sectioned for biochemical evaluation and histopathological analysis. The organosomatic index (OSI) of the testes was computed using this formula: OSI = [organ weight (g)/body weight (g)] × 100.

2.5 | Sperm motility assay

Sperm motility was assessed using a standard protocol (Zemjanis, 1970). Tersely, epididymal spermatozoon from the epididymis was harvested and deposited on clean glass slide, cautiously mixed with 2.9% sodium citrate dehydrate solution and thereafter enclosed with a coverslip. Assessment of sperm motility was performed by scoring the number of progressive, non progressive and motionless sperm cells using a Carl Zeiss AX10 microscope (magnification 200×).

2.6 | Calculation of sperm number

The epididymal sperm number calculated as previously reported (WHO 1999). Tersely, caudal epididymis was crushed in normal saline and sieved through a nylon mesh to obtain the spermatozoon. Thereafter, 95 µl of a diluent (formalin containing, $NaHCO_3$ and Trypan blue) was cautiously added to 5 µl of the spermatozoon. The number of spermatozoa was evaluated using a Carl Zeiss AX10 light microscope, hemocytometer and Neubauer chamber.

2.7 | Assessment of sperm viability and abnormalities

Viability of spermatozoa and morphological aberrations were assessed as previously described (Wells & Awa, 1970). Tersely, sperm smear was prepared on a clean glass slide by carefully spreading some sperm suspension with another slide. Sperm viability was assessed by staining the smeared slide with eosin (1%) and nigrosine (5%) in 3% sodium citrate dehydrate solution while sperm abnormalities were assessed using a eosin and fast green staining reagent in a ratio of 0.2 g to 0.6 g respectively.

2.8 | Assessment of reproductive hormones

Serum levels of FSH (RPN 2560), LH (RPN 2562) and testosterone (EIA-5179) were assessed using appropriate ELISA kits from

Amersham, UK, and DRG Diagnostics GmbH, Marburg, Germany, respectively, as described by the manufacturer in their product information. The sensitivities of the hormones were as follows: FSH (0.05 ng at 95%), LH (0.08 ng at 90%) and testosterone (0.04 ng/ml). The intra-assay coefficients of variations were FSH (3.4%), LH (3.1%) and testosterone (3.5%). Inter-assay variation was avoided by assessing all the hormones simultaneously.

2.9 | Evaluation of antioxidant and oxidative stress parameters

Samples (testes) were homogenised in 50 mM Tris–HCl buffer (pH 7.4) containing potassium chloride (1.15%). Homogenates were then centrifuged (12,000 *g* for 15 min at 4°C), and the resulting supernatant were stored for biochemical analyses. Assessment of total protein concentration was evaluated as described by Bradford (1976). Superoxide dismutase (SOD) was assessed as previously described by Misra and Fridovich (1972), in addition to the assessment of catalase (CAT) activity Clairborne (1995). Glutathione peroxidase (GPx) activity was assessed in line with Rotruck et al (1973). Glutathione-S-transferase (GST) activity was assessed in line with Habig, Pabst, and Jakoby (1974). Glutathione (GSH) was assessed by the method of Jollow, Mitchell, Zampaglione, and Gillette (1974), and malondialdehyde (MDA) levels, a marker of lipid peroxidation, were assessed according to the method of Farombi, Tahnteng, Agboola, Nwankwo, and Emerole (2000).

2.10 | Histopathological examination

Testes samples were processed for histology using Bouin's solution as a fixative according to standard protocol (Bancroft & Gamble, 2008). The tissue was then dehydrated in alcohol (graded concentrations), cleared in xylene and embedded in paraffin. Subsequently, sections of the tissue were prepared and stained with haematoxylin and eosin (H & E). Pathologists who were not aware of the treatment administered to experimental animals observed and scored the stained slides using a Carl Zeiss AX10 light microscope.

2.11 | Statistical analysis

Results were analysed using the one-way analysis of variance (ANOVA) and post hoc Bonferroni's test with the aid of GRAPHPAD PRISM 5 (GraphPad Software, La Jolla, CA, USA) to ascertain significant differences in the treatment groups. p values <0.05 were considered to be significant.

3 | RESULTS

3.1 | Impact of separate and combined administration of DEN and Cd on absolute and organo-somatic index of the testes

The absolute and organo-somatic index of testes of pubertal rats either singly or co-treated treated with DEN and Cd for 7 consecutive days is presented in Table 1. Separate and co-administration of DEN and Cd did not significantly affect the body weight gain compared with control (data not shown). Separate administration of DEN had no effect on absolute weight and OSI of the testes, whereas Cd exposure decreased ($p < 0.05$) the absolute weight and OSI of the testes. However, co-exposure to DEN and Cd markedly decreased the absolute weight and OSI of the testes compared to rats treated with DEN alone and Cd alone.

3.2 | Impact of separate and combined administration of DEN and Cd on sperm functional characteristics

Figure 1 shows the noxious effects of DEN alone, Cd alone and the combined treatment on sperm parameters. The epididymal sperm number and sperm progressive motility were decreased ($p < 0.05$) with concomitant increase ($p < 0.05$) in abnormal spermatozoon after oral administration of DEN alone or Cd alone to pubertal rats. The adverse effect was more evident in rats co-treated with DEN and Cd. Moreover, while the sperm viability was not affected ($p > 0.05$) in DEN alone group, there was a decrease ($p < 0.05$) in the sperm viability in Cd alone treated rats compared with control. The decrease sperm viability was intensified in rats concomitantly exposed to DEN and Cd.

3.3 | Impact of separate and combined administration of DEN and Cd on serum hormone levels

Figure 2 shows the impact of separate and concomitant exposure to DEN and Cd on serum FSH, LH, and testosterone levels in rats. In comparison with the control rats, separate administration of DEN and Cd did not alter ($p > 0.05$) the level of LH, whereas it decreases

TABLE 1 Absolute testes weight and organo-somatic index (OSI) of testes of pubertal rats following treatment with DEN alone, Cd alone and DEN + Cd for seven consecutive days

	Control	DEN alone	Cd alone	DEN + Cd
Absolute testes weight (g)	1.06 ± 0.14	0.87 ± 0.03*	0.52 ± 0.08*,**	0.46 ± 0.05*,**,***
OSI of the testes	0.82 ± 0.06	0.67 ± 0.04*	0.38 ± 0.04*,**	0.31 ± 0.06*,**,***

Note. Values are expressed as mean ± *SD* of ten rats.
*$p < 0.05$ versus Control. **$p < 0.0.0.05$ versus DEN alone. ***$p < 0.0.05$ versus Cd alone.

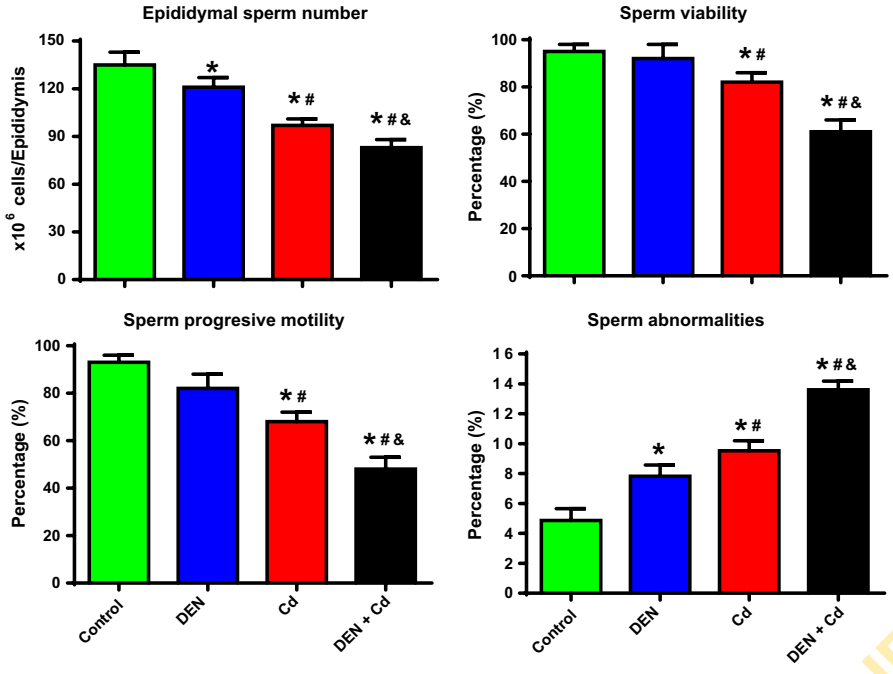

FIGURE 1 Epididymal sperm count, motility, viability and abnormalities in experimental rats following oral treatment with DEN alone, Cd alone and both DEN and Cd for 7 consecutive days. Each bar represents mean ± *SD* of ten rats. *$p < 0.05$ vs. Control; #$p < 0.0.01$ vs. DEN alone; &$p < 0.001$ vs. Cd alone

($p < 0.05$) FSH and testosterone levels compared with control. Decreases in FSH and testosterone levels were intensified in the rats treated with DEN and Cd.

3.4 | Separate and combined administration of DEN and Cd increased biomarkers of oxidative stress in rat testes

Figures 3–5 show the influence of separate and combined exposure to DEN and Cd on antioxidant defence system and indicators of oxidative stress. Administration of DEN or Cd alone triggered a marked decrease in testicular antioxidant status, whereas they markedly elevated biomarker of lipid peroxidation in the testes of rats compared with the control. Precisely, activities of testicular SOD, CAT, GST and GPx as well as GSH level were markedly decreased following separate administration of DEN and Cd but were increased in rats co-exposed to DEN and Cd. However, the significant increase in testicular MDA level in rats administered DEN alone or Cd alone was intensified in rats co-exposed to DEN and Cd.

3.5 | Histopathological changes in rat testes exposed to DEN and Cd

Figure 6 depicts representative photomicrographs of rat testes from the experimental groups. Testes from control rats showed testicular architecture with normal seminiferous tubules and epithelium consisting of spermatogonia, spermatocytes, spermatids, spermatozoa, Sertoli and Leydig cells. Testes from DEN-treated rats show seminiferous tubules and epithelium somewhat similar to control. Testes from Cd-treated rats showed general disruption of spermatogenesis and tubular microanatomy without visible terminally differentiated cells. These histopathological alterations observed in DEN alone and Cd alone were intensified in testes of rats co-treated with DEN and Cd.

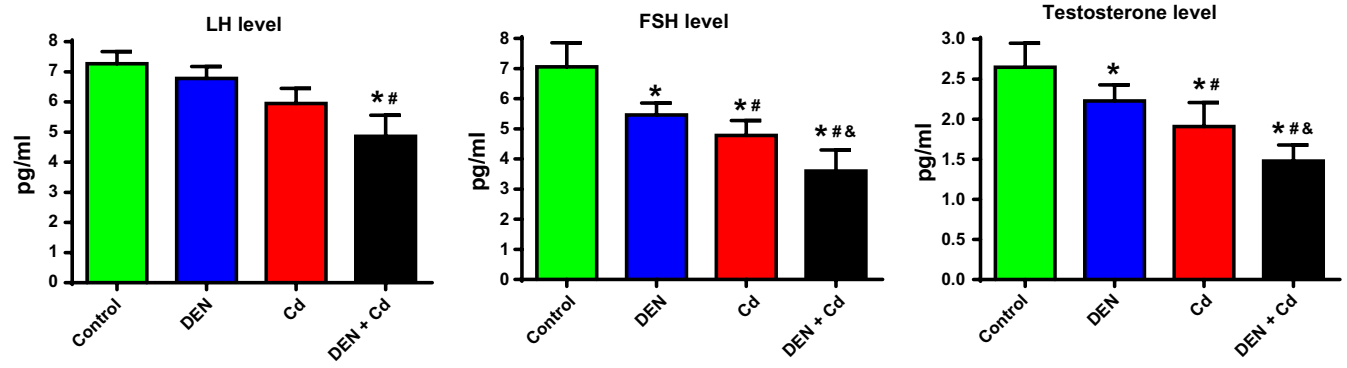

FIGURE 2 Circulatory concentrations of follicle-stimulating hormone (FSH), luteinising hormone (LH) and testosterone in experimental rats following oral treatment with DEN alone, Cd alone and both DEN and Cd for 7 consecutive days. Each bar represents mean ± *SD* of ten rats. *$p < 0.05$ vs. Control; #$p < 0.0.01$ vs. DEN alone; &$p < 0.001$ vs. Cd alone

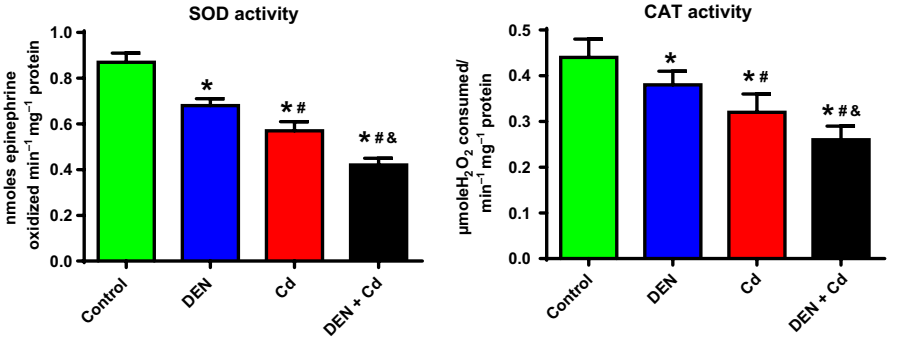

FIGURE 3 Activities of SOD and CAT in testes of rats following oral treatment with DEN alone, Cd alone and both DEN and Cd for 7 consecutive days. Each bar represents mean ± *SD* of ten rats. *$p < 0.05$ vs. Control; #$p < 0.0.01$ vs. DEN alone; &$p < 0.001$ vs. Cd alone

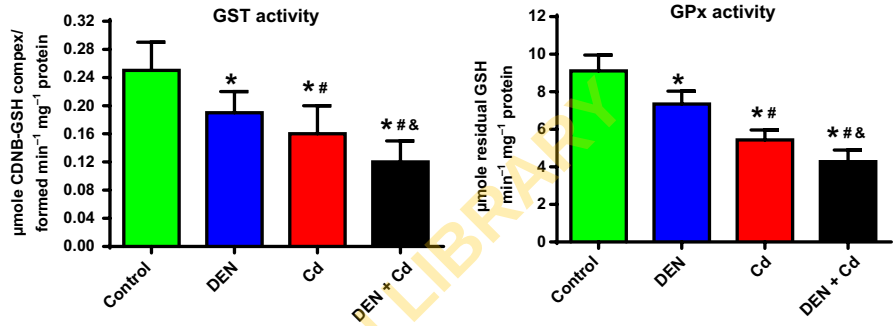

FIGURE 4 Activities of GST and GPx in testes of rats following oral treatment with DEN alone, Cd alone and both DEN and Cd for 7 consecutive days. Each bar represents mean ± *SD* of ten rats. *$p < 0.05$ vs. Control; #$p < 0.0.01$ vs. DEN alone; &$p < 0.001$ vs. Cd alone

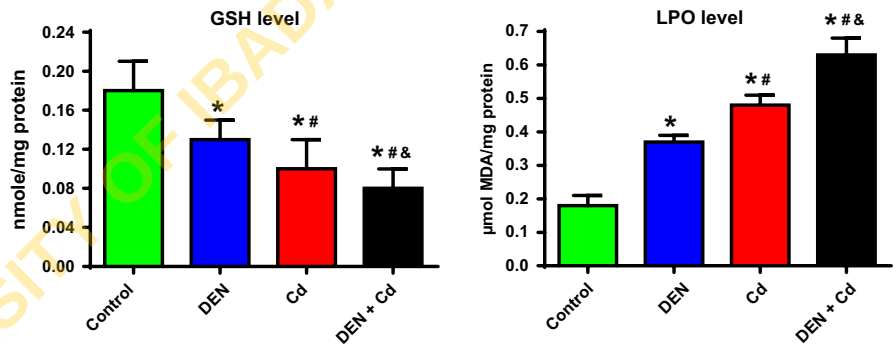

FIGURE 5 Levels GSH and LPO in testes rats following oral treatment with DEN alone, Cd alone and both DEN and Cd for 7 consecutive days. Each bar represents mean ± *SD* of ten rats. *$p < 0.05$ vs. Control; #$p < 0.0.01$ vs. DEN alone; &$p < 0.001$ vs. Cd alone

4 | DISCUSSION

Novel approaches for assessing risks associated with real-life exposures to chemical mixtures are currently of interest to researchers because systemic chemical interaction may result in antagonistic, synergistic or additive effects (Carpenter, Arcaro, & Spink, 2002) detrimental to health and well being. The potential toxic effects associated with co-exposure to dietary DEN and Cd, a ubiquitous environmental contaminant, are of great importance to human risk assessment. Herein, we assessed functional alterations in endocrine balance, sperm characteristics and antioxidant defence mechanisms in testes of pubertal rats acutely exposed to DEN and Cd. The weights of testes and other accessory organs are good indices of reproductive health (Adedara et al., 2016; Archibong et al., 2008). In the present study, the body weight gain were unchanged across all treatment groups, whereas decreases in the absolute and OSI of testes were aggravated in the rats co-administered DEN and Cd, thus revealing inhibition of normal function of the reproductive organ perhaps due to testicular damage in treated rats. These observations signify a graver toxic consequence of DEN and Cd co-exposure probably stemming from DEN and Cd interactions in the treated rats.

Epididymal sperm count is a major index for assessing spermatogenesis since it reveals completely product of cell division phases, spermiogenesis and movement within the epididymis (Chandra, Ghosh, Chatterjee, & Sarkar, 2007). Reduction in epididymal sperm amount observed in rats exposed to either DEN or Cd was intensified in the co-exposed rats. The reduction in sperm count may be associated with decrease testosterone bioavailability in treated rats. Moreover, sperm progressive motility and viability are functional parameters for sperm fertilising ability (WHO 1999). The decrease in sperm count, viability and motility with concomitant increase in abnormalities in rats co-exposed to DEN and Cd indicates spermatotoxicity which could impair fertilisation of ova. Hence, acute

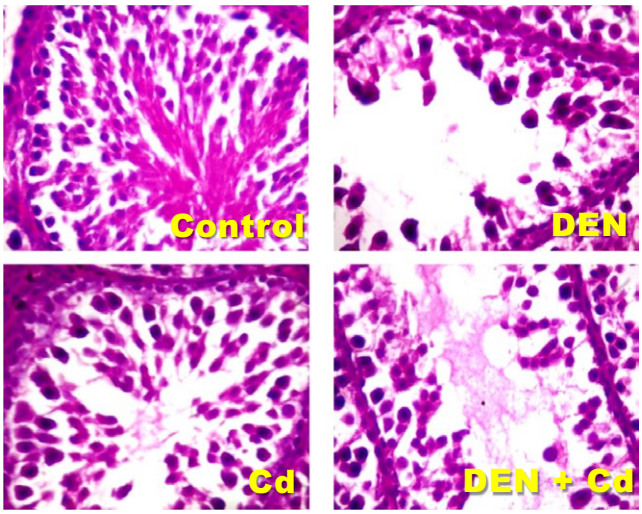

FIGURE 6 Representative photomicrographs of testes from control, DEN alone, Cd alone and co-exposure group. Control testes showed normal morphology. DEN-treated rats demonstrated seminiferous tubules and epithelium somewhat similar to control whereas Cd-treated rats showed general disruption of spermatogenesis and tubular microanatomy. Testes of rats co-treated with DEN and Cd showed excessive testicular degeneration

co-exposure to DEN and Cd resulted in an apparent greater risk on sperm parameters when compared with separate exposure to DEN or Cd.

Separate exposure of rats to DEN and Cd did not affect ($p > 0.05$) the serum LH level, whereas it triggered decreases ($p < 0.05$) in testosterone and FSH levels in the exposed rats. The observed decrease of FSH, LH and testosterone in rat's co-exposure to DEN and Cd reflects additive effects of treatment on endocrine function specifically along the hypothalamic–pituitary–testicular bloc. The pituitary gonadotropin LH is the major regulator of Leydig cells production and secretion of testosterone (Adedara, Nanjappa, Farombi, & Akingbemi, 2014). The marked reduction in testosterone level in serum signifies that DEN and Cd may impede steroidogenesis in the testis, which may be attributed to reduced Leydig cells function in rats exposed to DEN and Cd. The impairment in the function of the hypothalamus–pituitary–testicular axis may subsequently suppress testicular function of spermatogenesis.

In the present investigation, there were marked reductions in testicular CAT and SOD activities as a consequence of separate administrations of DEN and Cd to rats and in the co-exposure group. The conversion of deleterious superoxide radicals to H_2O_2 by SOD and the subsequent conversion of the noxious H_2O_2 into water and oxygen by CAT represent the primary defence against cellular oxidative damage (Adedara, Teberen, Ebokaiwe, Ehwerhemuepha, & Farombi, 2012). Increases in antioxidant enzymes activities in rats co-treated with DEN and Cd signify a graver inhibition in the animals' antioxidant defence role to testicular oxidative damage. Glutathione, a non enzymatic antioxidant, protects against oxidative stress by maintaining the intracellular redox status and xenobiotic detoxification pathway in the cell (Rana, Allen, & Singh, 2002). Antioxidant enzyme inhibition and reduction of GSH level are associated with cellular oxidative stress. The exacerbating noxious effects of co-exposure to DEN and Cd was evidenced by the marked reduction in the testicular GSH and GSH-dependent enzymes (i.e., GST and GPx) levels, with concomitant increase in MDA, a biomarker of lipid peroxidation in the treated rats when compared with DEN alone and Cd alone groups. Histological analysis showed that impairment of the germ cells maturation and the absence of terminally differentiated sperm cells were aggravated in testes of rats co-exposed to DEN and Cd, thus further confirming the toxicity of DEN and Cd co-exposure in pubertal rats.

Conclusively, the results from this investigation indicate that DEN and Cd co-exposure occasioned a more noxious effects than the individual exposure owing to the additive toxic impact on testicular functions in pubertal rats. The findings in this study are new and demonstrated the apparent link between chemical interactions and testicular dysfunction in pubertal rats. The joint testicular damage by DEN and Cd is associated with suppression of endocrine and redox regulatory mechanisms in rats so exposed.

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CONFLICT OF INTEREST

In the present investigation, the authors are not aware of any conflicts of interest and so declare no conflicts of interest.

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