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Research Article
Evidences for Spermatozoa Toxicity and Oxidative Damage of Cadmium Exposure in Rats

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Abstract

Background: Spermatogenesis is a complex series of differentiation process that can be interfered by toxic chemicals, heavy metals, heat, radiation, deficiencies of hormones and immunodeficiency. **Materials and Methods:** Fifteen male Wistar rats (10-12 weeks old) weighing 164-279 g were divided into three groups of four rats each. The rats in group A served as the control group and were treated with 2.5 mL kg^{-1} b.wt., daily, group B and C rats were given a single dose of 5 and 7 mg kg^{-1} b.wt., of cadmium chloride solution intraperitoneally. The protocol lasted for 8 weeks. **Results:** There was a significant ($p>0.01$) reduction in spermatozoa count, spermatozoa motility, percentage number of morphologically normal spermatozoa and a significant ($p>0.01$) increase in the percentage number of morphologically abnormal spermatozoa (headless sperm, rudimentary tail, curved tail, curved mid-piece) in animal models exposed to cadmium. There was a significant ($p<0.05$) difference in SOD, GSH, CAT and MDA activity in the groups of rats when compared to the negative control group. **Conclusion:** This study has shown that cadmium induces morphological and functional abnormalities on rat spermatozoa by reducing antioxidant status.

Key words: Cadmium, testes, oxidative stress, infertility, spermatozoa

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cadmium is used traditionally in pigments, coatings and stabilizer production[1]. The reproductive potential of species and their survival have been threatened by an increased industrial and environmental contamination[2]. Cadmium is also found naturally in tobacco leaves[3] hence cigarettes smoking is a sure way to cadmium exposure[3,4] of upto 1.7 μg cigarette^{-1}.

Cadmium is a potent heavy metal carcinogen to animals[5] and humans[6]. The gonads, ventral prostate, liver and kidney are target sites for cadmium toxicity in rodents[7-11]. It has been reported to exert its genotoxicity via the production of reactive oxygen species and by inhibiting cell proliferation and DNA replication[9].

Cadmium cytotoxicity depends majorly on ionic mimicry distinct by calcium and zinc substitution which results in protein breakdown and ultimately endoplasmic reticulum stress and cell death. Several studies indicating the exact progression of mitochondrial dilapidation as a result of its cadmium toxicity has been reported. These includes opening of the mitochondrial permeability transition pore (mPTP) and Mitochondrial Calcium Uniporter (MCU) thereby leading to the release of cytochrome C into the cytosol.

Unlike somatic cells, mammalian sperm cells present unusually high specific lipidic composition with high content of poly-unsaturated fatty acids, plasmalogenes and sphingomyelins responsible for its flexibility and the functionality. However, these lipids are substrates for peroxidation that may provoke severe functional disorder of sperm[12].

The aim of this study was to evaluate the spermatotoxic effects of low dose cadmium exposure in rat models and the possible mechanism of action.

MATERIALS AND METHODS

Chemicals and reagents: Analytical grade cadmium chloride ($CdCl_2$) with 96% purity was obtained from Department of chemistry, Federal University Ndufu Alike Ikwo, Ebonyi State, Nigeria.

Experimental protocol: Fifteen Wistar rats (10-12 weeks old) weighing 164-279 g were secured from the animal house of the Department of physiology, University of Nigeria, Enugu Campus. The animals were housed in well ventilated wire cages in the Animal Facility and an ethical approval was obtained from the Ethical Committee on Animal use with reference NNHREC/05/01/2008b-FWA00002458-1RB00002323.

The rats were divided into three groups of four rats each. The rats in group A served as the control group and were treated with 2.5 mL kg^{-1} b.wt., daily, group B and C rats were given a single dose of 5 and 7 mg kg^{-1} b.wt., of cadmium chloride solution intraperitoneally[13,14]. The protocol lasted for 8 weeks (Duration of spermatogenesis in rat being 52-56 days[15].

Animal sacrifice and sample collection: The rats were first weighed and then sacrificed by cervical dislocation. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. The testes were excised and trimmed of all fat. The testicular weights of each animal were evaluated with an electronic analytical and precision balance (BA 210S, d = 0.0001-Sartoriusen GA, Goettingen, Germany). The testes volumes were measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. One of the testes of each animal was fixed in boun's fluid for histological and morphometric analysis. Serum and the remaining testes of each animal were stored at -25°C for subsequent biochemical assays.

Epididymal sperm counts, motility and morphology: Spermatozoa progressive motility was evaluated with the method described by Sonmez *et al.*[16]. The fluid obtained from the left cauda epididymis with a pipette was diluted to 0.5 mL with tris buffer solution (pH 7.5). A slide was placed on light microscope with heater table; an aliqout of this solution was on the slide. The microscopic field was scanned systematically and each spermatozoon encountered was assessed and for the purpose of the first part of the assessment, motility was classified as either motile or non-motile and percentage motility was evaluated visually at a magnification of x400. Motility estimates were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score. Samples for motility evaluation were be incubated at 35°C. Further, motile spermatozoa was classified as either rapid linear progressive or sluggish non-linear motile.

Spermatozoa in the right epididymis were counted by a modified method of Yokoi and Mayi[17]. Briefly, the epididymis was minced with anatomic scissors in 5 mL physiologic saline, placed in a rocker for 10 min and allowed to incubate at room temperature for 2 min. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5 g sodium bicarbonate and 1 mL formalin (35%). Total sperm number was determined by using the new improved Neubeur`s

counting chamber (haemocytometer). Approximately 10 µL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and was allowed to stand for 5 min. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was focused and the number of spermatozoa counted in five 16 celled squares. The sperm concentration was the calculated number multiplied by 5 and expressed as $[X] \times 10^6$ mL^{-1}, where, [X] is the number of spermatozoa in a 16-celled square.

Normal and abnormal spermatozoa morphology were evaluated with the aid of light microscope at x400 magnification. Caudal sperm will be taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada). Five hundred sperm from the sample were scored for morphological abnormalities[18]. Briefly, in wet preparations using phase-contrast optics, spermatozoa were categorized. In this study a spermatozoon was considered abnormal morphologically if it had one or more of the following features: Rudimentary tail, round head and detached head and will be expressed as a percentage of morphologically normal sperm.

Assay of testicular enzymatic antioxidants

Catalase (CAT) activity: Catalse activity was measured according to the method of Aebi[19]. Testicular homogenate (0.1 mL) was pipetted into cuvette containing 1.9 mL of 50 mM phosphate buffer, pH 7.0. Reaction was started by the addition of 1.0 mL of freshly prepared 30% (v/v) hydrogen peroxide (H_2O_2). The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of enzyme was expressed as µ mg^{-1} protein.

Superoxide dismutase (SOD) activity: Superoxide dismutase activity was measured according to the method of Winterbourn *et al.*[20] as described by Rukmini *et al.*[21]. The principle of the assay was based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT). Briefly, the reaction mixture contained 2.7 mL of 0.067 M phosphate buffer, pH 7.8, 0.05 mL of 0.12 mM riboflavin, 0.1 mL of 1.5 mM NBT, 0.05 mL of 0.01 M methionine and 0.1 mL of enzyme samples. Uniform illumination of the tubes was ensured by placing it in air aluminum foil in a box with a 15 W fluorescent lamp for 10 min. Control without the enzyme source was included. The absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction of NBT by 50% under the specific conditions. Activity of enzyme was expressed as µ mg^{-1} protein.

Assay of testicular non-enzymatic antioxidants

Reduced glutathione (GSH) concentration: The GSH was determined by the method of Ellman[22]. Testicular homogenate-supernatant (0.1 mL) was treated with 0.5 mL of Ellmans reagent (19.8 mg of 5, 5′-dithiobisnitro benzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0). Distilled water (0.4 mL) was added. The mixture was thoroughly mixed and the absorbance was read at 412 nm. Reduced glutathione concentration was expressed as nmol mg^{-1} protein.

Lipid peroxidation (Malondialdehyde): Lipid peroxidation in the testicular tissue was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) method of Buege and Aust[23]. A principle component of TBARS being malondialdehyde (MDA), a product of lipid peroxidation. In brief, 0.1 mL of tissue homogenate in tris-HCl buffer, pH 7.5 was treated with 2 mL of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was calculated using the molar absorptivity of malondialdehyde which is 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol mg^{-1} protein.

Statistical analysis: The data were statistically analyzed and expressed as Mean±SD. Analysis was carried out using analysis of variance (ANOVA) with Scheffe's *post hoc* test. The level of significance was considered at $p<0.05$ and $p<0.01$.

RESULTS AND DISCUSSION

Spermatogenesis is a complex series of differentiation process that can be interfered by toxic chemicals, heavy metals, heat, radiation, deficiencies of hormones and immunodeficiency[24-26]. In this study, results show a significant ($p>0.01$) reduction in spermatozoa count, spermatozoa motility, percentage number of morphologically normal spermatozoa and a significant ($p>0.01$) increase in the percentage number of morphologically abnormal spermatozoa (headless sperm, rudimentary tail, curved tail and curved mid-piece) in animal models exposed to cadmium, when compared to control group (Table 1, 2, Fig. 1-3). Our results are in line with several other studies[25-28]. Studies has shown the degenerative ability of cadmium to testicular and epididymal tissues thereby contributing to male infertility by reducing sperm quality in humans and rats[26,28-35]. Although the effect of cadmium has been reported to be dose dependent on testicular tissues[35,29], our result on sperm characteristics were not in accordance with this line of thought.

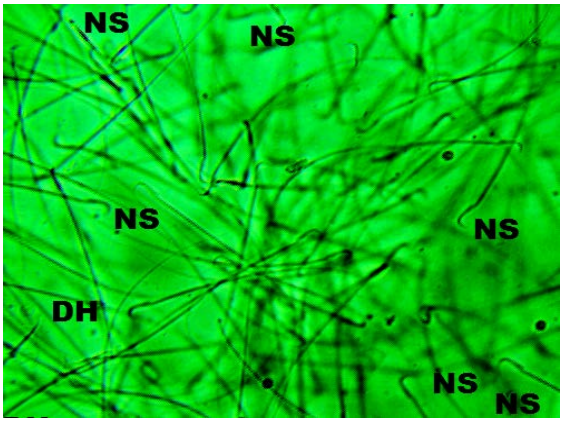

Fig. 1: Photomicrograph of sperm from group A rats (Treated with a single dose of 2.5 mL kg^{-1} b.wt., of normal saline). Showing spermatozoa with normal morphology. RH: Round head, DH: Detached, RT: Round tail, AT: Abnormal tail, NS: Normal sperm, magnification: x100

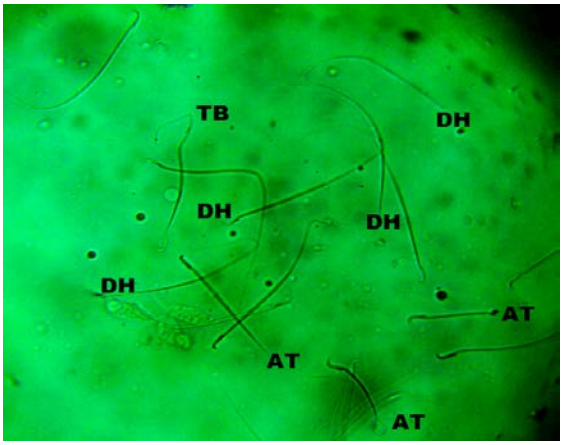

Fig. 2: Photomicrograph of sperm from group B rats (Treated with a single dose of 5 mg kg^{-1} b.wt., of cadmium) showing numerous abnormal spermatozoa. DH: Detached head, TB: Twisted body, AT: Abnormal tail, magnification: x100

Cadmium has been reported to cause spermatotoxicity either by resulting in disruption of hypothalamic-pituitary axis or by direct effect on spermatogenesis through oxidative damage[27]. Since sperm cell and testicular leydig cell mitochondria are common body cells that are susceptible to cadmium-induced oxidative stress[26,36,37], the latter represents the major factor. This was validated by our results on testicular enzyme activities of SOD, CAT, GSH and MDA compiled in Table 3.

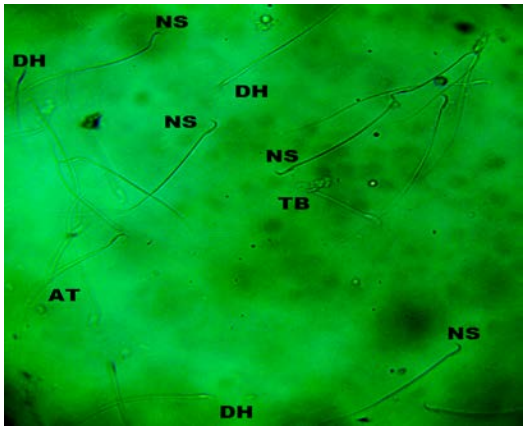

Fig. 3: Photomicrograph of sperm from group C (Treated with a single dose of 7 mg kg^{-1} b.wt., of cadmium) showing mostly abnormal spermatozoa. RH: Round head, DH: Detached, AT: Abnormal tail, NS: Normal sperm, magnification: x100

Table 1: Effects of cadmium on epididymal spermatozoa concentration ($\times 10^6$ mL^{-1}) spermatozoa motility (%) and spermatozoa morphology (Head) in Wistar rats

Treatment groups	Sperm count ($\times 10^6$ mL^{-1})	Motile sperm (%)	Sluggish sperm (%)	Immotile sperm (%)	Headless sperm (%)
Group A	134.2±9.9	76.1±1.4	15.5±0.89	23.9±1.8	7.0±2.1
Group B	28.2±7.3**	41.1±2.1**	20.4±2.1*	58.9±3.2**	17.2±9.1**
Group C	27.1±1.1**	29.3±1.4**	29.4±2.1**	70.7±0.5**	19.0±2.3**

*,**Represent significant increases or decreases at $p<0.05$ and $p<0.01$ respectively, when compared to negative control (Group A). Values are Means±SD, n = 5 in each group. Group A: Single dose of 2.5 mL kg^{-1} b.wt., of normal saline, group B: Single dose of 5 mg kg^{-1} b.wt., of cadmium, group C: Single dose of 7 mg kg^{-1} b.wt., of cadmium

There was a significant ($p<0.05$) difference in SOD, GSH, CAT and MDA activity in the groups of rats when compared to the negative control group (Table 3). Our results were in line with several other results on cadmium-induced oxidative stress[11,38-42]. In 2011, Bu *et al.*[2] reported a significant increase in lipid peroxidation and reduction in GSH, SOD, CAT and GSH activity level[38,11,39,43-45].

In the cell, cadmium forms complexes with thiol residues from the tripeptide-reduced glutathione (GSH). This is thought to be the first line of defense. As a thiol-affectionate metal, cadmium mainly targets cellular GSH thereby causing a depletion that leads to reduced scavenging of cadmium and cellular redox balance hence oxidative stress[46,47]. Although cadmium a non-fenton metal is incapable of inducing reactive oxygen specie by itself[48], it can however induce oxidative stress directly by a displacement of redox-active metals, diminution of redox scavengers, inhibition of anti-oxidant enzymes and inhibition of the electron transport chain thereby causing mitochondrial damage[46,47,49]. Cadmium can

Table 2: Effects of cadmium on spermatozoa morphology (tail and mid-piece) in Wistar rats

Treatment groups	Rudimentary tail (%)	Curved mid-piece (%)	Curved tail (%)	Total abnormal (%)	Total normal (%)	Total cell counted (%)
Group A	6.5±6.3	14.3±0.1	4.4±2.0	32.2±5.2	460±3.1	492.2±0.7
Group B	20.1±3.2**	29.2±2.2**	22.1±2.3**	88.6±2.5**	187.2±0.4**	275.8±1.1**
Group C	17.4±0.5**	32.0±0.2**	26.7±1.1**	94.4±8.1**	173.1±9.1**	267.5±2.0**

*,**Represent significant increases or decreases at p<0.05 and p<0.01 respectively, when compared to negative control (Group A). Values are Means±SD, n = 5 in each group. Group A: Single dose of 2.5 mL kg^{-1} b.wt., of normal saline, group B: Single dose of 5 mg kg^{-1} b.wt., of cadmium, group C: Single dose of 7 mg kg^{-1} b.wt., of cadmium

Table 3: Effects of cadmium on the activities of testicular superoxide dismutase (SOD), testicular glutathione (GSH), catalase (CAT) and lipid peroxidation (MDA)

Treatment groups	SOD (μ mg^{-1} protein)	GSH (μ mg^{-1} protein)	CAT (μ mg^{-1} protein)	MDA (nmol mg^{-1})
Group A	44.3±2.6	2.5±0.3	15.5±1.9	0.3±0.1
Group B	17.9±0.5*	1.0±0.1*	8.7±0.2*	2.1±0.04*
Group C	15.3±1.6**	1.2±0.5*	9.9±3.0*	2.9±0.6*

*,**Represent significant increases or decreases at p<0.05 and p<0.01 respectively when compared to negative control (Group A). Values are Means±SD, n = 5 in each group. Group A: Single dose of 2.5 mL kg^{-1} b.wt., of normal saline, group B: Single dose of 5 mg kg^{-1} b.wt., of cadmium, group C: Single dose of 7 mg kg^{-1} b.wt., of cadmium, SOD: Superoxide dismutase, GSH: Testicular glutathione peroxidase, CAT: Catalase, MDA: Malondialdehyde

replace Fe, a redox-active metal, hence escalating the availability of liberated Fe in cells thereby inducing oxidative stress and in turn through the Fenton reaction, produces extremely detrimental hydroxyl radicals (•OH)[46-48].

Although cadmium cannot produce free radicals in fenton type chemistry, however, it can lead to oxidative stress via a multifaceted mechanism which includes the reduction of in-built antioxidative defense and the stimulation of mass production of reactive oxygen species which is of course through mitochondrial damage.

Adenosine triphosphate (ATP) is an energy source for sperm motility and its unavailability may be a limiting factor responsible for loss of sperm motility in cadmium-treated rats[50]. This could explain the significant reduction in sperm concentration, sperm motility and normal sperm morphology along with a significantly increased abnormal sperm morphology rates as seen in cadmium treated group rats when compared to the control groups.

CONCLUSION

- In terms of biochemical results, this study has provided an addition to the body of knowledge by extensively reporting the oxidative mechanism pathway of cadmium induced spermatoxicity through assay of testicular enzymatic antioxidants and testicular non-enzymatic antioxidants
- To the best of our knowledge this study is significantly different from those already published in that we provided the first extensive rat sperm morphological characteristics of rat spermatozoa after cadmium exposure

SIGNIFICANCE STATEMENTS

- In terms of biochemical results, this study has provided an addition to the body of knowledge by extensively reporting the oxidative mechanism pathway of cadmium induced spermatoxicity through assay of testicular enzymatic antioxidants and testicular non-enzymatic antioxidants
- To the best of our knowledge this study is significantly different from those already published in that we provided the first extensive rat sperm morphological characteristics of rat spermatozoa after cadmium exposure

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