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N-acetylcysteine and zinc sulphate abate di-2-ethylhexyl phthalate-mediated reproductive dysfunction in rats: Focus on oxidative and sex hormone receptors mechanisms

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ABSTRACT

Objective: To investigate the potential of *N*-acetylcysteine (NAC) and zinc sulphate (ZnSO₄) in mitigating reproductive dysfunction caused by di-2-ethylhexyl phthalate (DEHP) in rats and to understand the underlying mechanisms, specifically oxidative stress and sex hormone receptor activity.

Methods: Thirty-five male Wistar rats were randomly divided into five equal groups ($n=7$ per group). Group 1 was administered 0.5 mL of distilled water and served as the control group. Group 2 was given only DEHP (750 mg/kg/day), while group 3, 4 and 5 were given DEHP (750 mg/kg/day) plus NAC (100 mg/kg/day), DEHP (750 mg/kg/day) plus ZnSO₄ (0.5 mg/kg/day), and DEHP (750 mg/kg/day) plus NAC (100 mg/kg/day) as well as ZnSO₄ (0.5 mg/kg/day), respectively. All treatments lasted for 21 days. Samples were obtained after the rats were sacrificed, and hormones levels in the serum and markers of oxidative stress in the testicles were analyzed using the enzyme-linked immunosorbent assay. The amount of androgen receptors in the testicles was determined by immunohistochemistry, and the susceptibility of testosterone and DEHP to bind to androgen receptor and 5 α -reductase was determined by molecular docking studies.

Results: DEHP decreased reproductive hormones, testicular antioxidant enzymes, increased malondialdehyde levels, and negatively impacted histology of the pituitary and testes. NAC or ZnSO₄ treatment showed a marked improvement in testicular antioxidant status and hormone levels, as well as a positive effect on the histology of the pituitary and testes. The combination of both treatments appeared to be more effective. The affinity of DEHP to bind to androgen receptors may lead to disruption of androgen receptor signaling, which can further result in dysfunction of hormones related to androgen. However, NAC is more likely to form stronger binding interactions with follicle stimulating hormone and

lutinizing hormone receptors, as well as gonadotropin-releasing hormone receptors, when compared to DEHP.

Conclusions: The possibility that NAC and ZnSO₄ could downregulate DEHP-induced sex hormone changes is suggested by their potential to reduce toxicity.

KEYWORDS: Di-2-ethylhexyl phthalate; Testosterone; Androgen receptor; 5 α -reductase; Oestrogen; Luteinizing hormone

Significance

The study demonstrates that *N*-acetylcysteine and zinc sulphate can effectively reduce reproductive dysfunction in rats caused by di-2-ethylhexyl phthalate, an endocrine disruptor that interferes with hormone receptors. The study also sheds light on the underlying mechanisms of oxidative stress and hormone receptor activity in this process. This research has implications for potential treatments for reproductive dysfunction in humans, as well as for understanding the harmful effects of environmental toxins on reproductive health.

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1. Introduction

Di-2-ethylhexyl phthalate (DEHP) is a low molecular weight phthalate that is usually used to make a variety of products, including medical equipment, food processing components, cosmetics, and personal care products[1,2]. It is mainly used in the manufacturing industry as a plasticizer. DEHP has been linked to endocrine disruption, which can interfere with the activity of the hypothalamic-pituitary-gonadal (HPG) axis. This disruption can cause hormones related to the reproductive system to be imbalanced, resulting in developmental and growth issues[3]. Under normal conditions, the HPG axis is the key player in controlling and regulating hormones that affect the functioning of the testes. Specifically, the hypothalamus secretes and releases gonadotropin-releasing hormone (GnRH) that hormone then stimulates the release of gonadotropins, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH)[4], from the anterior pituitary gland. Certain hormones are responsible for the regulation of gonadal activities, including the upkeep of spermatogenesis[5].

Endocrine disruptors such as DEHP can interfere with the HPG axis and result in changes to the levels of reproductive hormones in humans[6]. DEHP and other phthalates are found in food and food packaging, airborne particles, and contact with the skin, leading to increased lifetime exposure[1]. The effects of DEHP on human health are still largely unknown, but it has been suggested that DEHP can lead to adverse outcomes, including alterations in sex hormones, oxidative stress, and inflammation[2]. DEHP can cause adverse effects on male reproductive health[7,8]. Studies have shown that DEHP can interfere with testosterone production, leading to oxidative stress, and consequently causing disruptions to the reproductive organs of experimental animals[7,8]. Oxidative stress is known to be a significant factor in endocrine disorder development, particularly in testicular cells[9]. DEHP is believed to be an endocrine-disrupting chemical, and its toxic effects are thought to be attributed to its interaction with hormone transport proteins, and steroid hormone enzymatic pathways[10,11]. This is due to its capability to induce oxidative stress. Testicular tissues are especially vulnerable to oxidative stress, which is largely attributed to their high rate of cell division and the lower oxygen pressure owing to the diminished vessels. Moreover, the polyunsaturated fatty acids (PUFAs) also affect this vulnerability[12]. Therefore, toxicants that act as pro-oxidants and trigger lipid peroxidation can easily modify the hormone production, sperm index, sperm DNA integrity, and acrosome reaction[13]. A possible way to reduce the effect of DEHP exposure on the body is to inhibit the production of reactive oxygen species (ROS) and increase antioxidant activity. This could be accomplished through dietary antioxidant supplementation, such as consuming naturally occurring antioxidants like selenium, which can act as free radical scavengers and reduce cytotoxicity[6,14].

N-acetylcysteine (NAC) is an antioxidant that provides protection to cell components from oxidative stress, and helps to raise and support intracellular levels of glutathione. It has a free thiol group which binds to ROS, resulting in protective effects on many different cell types. NAC has been found to restore the balance between pro-oxidant and antioxidant agents during states of oxidative stress. Intake of NAC is generally safe and well-tolerated; however, intravenous administration has been linked to some health issues. Various studies have also confirmed NAC's antioxidative capacities, and its potential to counteract damage caused by toxicants. It has been hypothesized that the use of NAC may be able to protect against DEHP-induced reproductive toxicity due to its demonstrated effectiveness in protecting against other types of reproductive toxicity. This has been seen in a number of studies, such as the prevention of placental toxicity and teratogenicity caused by cisplatin in rats[15], the protection against cyclophosphamide-induced testicular toxicity in Wistar rats[16], and the prevention of fluoride-induced testicular apoptosis in rats[17].

Zinc sulphate (ZnSO_4) is a sulphate form of zinc, a trace element with an important role in organisms. It functions as an antioxidant, is present in small quantities in the cells and tissues of organisms, and aids in numerous cellular processes such as signal transduction, transcription, and replication. Approximately 3%-10% of all proteins in mammalian genomes require zinc for their activity. Studies have shown that ZnSO_4 can be protective against toxicant-mediated activities. Examples include aluminum sulphate in male rats[18], chronic copper administration in mice[19] and dibutyl phthalate-induced adverse reproductive effect in Sertoli cells[20]. However, there is no existing research on the single or combined effects of NAC and ZnSO_4 in regards to DEHP-induced reproductive impairment. To better understand the effects of DEHP on reproductive toxicity, a study was conducted in Wistar rats to evaluate the effects of NAC and ZnSO_4 on the biological impacts of DEHP exposure.

The current research focused on assessing the potential of NAC and ZnSO_4 to mitigate the risks of DEHP on sex hormones, oxidative stress, and other related health parameters. In addition to taking advantage of the power of computational biology in drug development, we also explored the molecular basis of the effects of DEHP on testosterone levels, as well as the drug-receptor binding and affinity, by assessing the binding affinity of DEHP for androgen receptor and 5α -reductase using *in silico* molecular docking[21].

2. Materials and methods

2.1. Collection and administration of drugs

DEHP and NAC used in this study were sourced from Sigma Aldrich (USA), and ZnSO_4 was purchased from Uche-care

Pharmaceuticals (Ondo). Enzyme-linked immunosorbent assay (ELISA) kits for measuring serum testosterone, oestrogen, and LH were obtained from Monobind Inc. (CA 92630, USA).

2.2. Experimental animal model

Male Wister rats (weighing 200–250 g, aged 8–10 weeks) were procured from the Animal House Department of the Delta State University in Abraka, Delta State, Nigeria. The rats were then housed and cared for at the Animal House of the Department of Physiology, Delta State University in Abraka, Delta State, Nigeria, where the research was conducted. The animals were held in a laboratory environment with 12 h of light and 12 h of darkness at a temperature of $(25 \pm 2)^\circ\text{C}$. They were provided with unlimited access to rat pellets of standardized composition and water. Each group of animals was placed in labeled cages and was given two weeks to acclimate before the start of the DEHP administration.

2.3. Experimental design

Thirty-five male Wistar rats were randomly divided into five equal groups ($n=7$). Group 1, the control, was administered 0.5 mL of distilled water (placebo). Group 2 was given only DEHP (750 mg/kg/day), while group 3 were given both DEHP (750 mg/kg/day) and NAC (100 mg/kg/day). Group 4 was provided with DEHP (750 mg/kg/day) and ZnSO_4 (0.5 mg/kg/day), and group 5 was administered DEHP (750 mg/kg/day) plus NAC (100 mg/kg/day) and ZnSO_4 (0.5 mg/kg/day). All treatments (groups 1–5) lasted for 21 days. DEHP was administered as a viscous fluid at a dosage of 750 mg/kg/day[22]. NAC was dissolved in distilled water and given to the animals orally with the aid of an oro-gastric cannula at a dose of 100 mg/kg body weight per day[23]. Additionally, ZnSO_4 was also dissolved in distilled water and administered to the animals at 0.5 mg/kg body weight per day[24].

2.4. Samples collection

Following a 21 day experimental period, animals were given an overnight fast before being euthanized with an injection of light thiopental sodium. Blood samples were then collected through ventricle punctures. After centrifugation, the serum could then be used for hormone and biochemical analysis. Additionally, the testes and pituitary gland were removed, weighed and analyzed for physiological and biochemical results. However, the tissues for histological studies were immediately preserved in buffered formalin.

2.5. Estimation of serum reproductive hormones

Levels of LH, estrogen and testosterone (Los Angeles, CA,

USA) were determined through commercially available ELISA kits[25,26]. Hormonal fertility measures the performance of the Leydig and Sertoli cells. This is done by assessing the levels of LH, testosterone, and oestrogen in the body. Once these hormones have been determined, the testosterone secretion indexes (T/LH) and testosterone–oestrogen (T/O) ratios can then be calculated by dividing the testosterone by LH and oestrogen levels, respectively. These results are important in assessing the impact of DEHP on the body.

2.6. Estimation of malondialdehyde (MDA)

MDA concentration was determined using a method described by Olaniyan *et al*[27]. MDA is a biomarker that correlates with the levels of lipid peroxidation and ROS. The serum samples were added to thiobarbituric acid and placed in a micro-centrifuge tube for incubation. After the incubation period, the samples were centrifuged at $2000 \times g$ for 10 min and the absorbance of the supernatant was measured at 532 nm in two separate samples. The lipid peroxidation of the DEHP's impact on the body was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) produced. MDA bis-(dimethyl acetal) were used as the external standard and the results were expressed as MDA per gram of wet tissue. The tissue samples were homogenized in 1 g of physiological saline (5 mL) according to Pirinççi *et al*[28], using a homogenizer from B. Braun Melsungen AG (Germany). The analysis of the testicular anti-oxidative stress enzymes was then done[26].

2.7. Estimation of testicular superoxide dismutase (SOD)

Testicular SOD was measured using a technique described by Madesh and Balasubramanian[29]. This method utilizes pyrogallolautooxidation to produce superoxide, and inhibits superoxide-dependent reduction of the tetrazolium dye MTT [3-(4-5 dimethyl thiazol 2-yl) 2,5-diphenyl tetrazolium bromide], which is then measured at 570 nm. The reaction was stopped by the incorporation of dimethyl sulfoxide (DMSO), which helps to dissolve the formazan produced. The color evolution was observed to be steady for numerous hours and was indicated as SOD units [one SOD unit is the amount (in one gram) of protein needed to reduce the MTT reduction by 50%].

2.8. Estimation of testicular catalase

The catalase activity in a sample was measured by looking at the reduction of hydrogen peroxide (H_2O_2) concentration that arose after a standard (H_2O_2) solution was incubated with the sample. This was done using a spectrophotometric method. To assess catalase activity, 10% of the homogenate was used. To measure the impact of DEHP on the body, 1.99 mL of phosphate buffer and 10 μL of homogenate

were first added to a test tube. The contents were then transferred to a cuvette, and 1 mL of H₂O₂ was added directly into the cuvette to begin the reaction. The optical density (OD) was recorded every 30 s for 3 min at 240 nm against distilled water (as a blank).

The activity of catalase was expressed as mM H₂O₂ utilized/min/mg protein and calculated using the formula:

$$\text{Catalase} = \frac{\text{DOD}}{0.067} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times \frac{1}{\text{mg protein} \times \text{time}}$$

Where DOD is change in absorbance.

2.9. Estimation of glutathione peroxidase (GPx)

GPx activity was assessed[30]. The mixture of 2.48 mL of phosphate buffer (pH 7.0) with ethylene diamine tetraacetic acid (EDTA), 0.1 mL nicotinamide adenine dinucleotide phosphate (NADPH), 0.1 mL GSH, 0.01 mL sodium azide, and 4.6 U glutathione reductase was prepared. The reaction was started by adding 0.1 mL of H₂O₂ to the mixture. The absorbance at 340 nm was monitored for 3 min. To measure enzyme activity, the molar extinction coefficient of 6200/M/cm at 340 nm was employed. Enzyme activity was measured in terms of μ moles of NADPH oxidized to NADP per milligram of protein per minute.

2.10. Estimation of testicular androgen receptor by immunohistochemistry

Immunohistochemistry, a procedure originally adopted by Zhu *et al*[31], was applied to androgen receptors on testicular tissues. The primary antibody used for the reaction was a polyclonal (rabbit) anti-androgen receptors (PA1-111A; Affinity Bioreagents Inc., Colorado, Golden, USA). Following the reaction, brown coloring was observed in the nuclei of Sertoli cells and Leydig cells as well as the areas of the seminiferous tubule. Mayer's hematoxylin was used as a counterstain. The negative control sections were prepared using the same process, except that instead of the primary antibody (PA1-111A), a phosphate buffered saline (PBS) buffer was used. A strong androgen immunoreaction was observed in both the nucleus and cytoplasm of the seminiferous tubule, Sertoli and Leydig cells, in the positive control tissue (radial testicular sections)[32].

The testicular tissue samples were prepared for analysis by first being embedded in a substance called O.C.T compound and then frozen in liquid nitrogen for storage at a temperature of 70 °C. Subsequently, sections of tissues with thicknesses of 6 to 7 mm were cut with a cryostat microtome at a temperature of 20 °C and mounted on glass slides that had been coated in a poly-L-lysine with a molecular weight of 150000.

Tissue samples were sectioned into 12 pieces. 8 Portions were used for androgen receptor immunostaining and the other 4 served as the controls. These parts were dried in room temperature and stored in 4% paraformaldehyde in PBS (pH 7.4) for 10 min. They were then

washed thoroughly with PBS before streptavidin-biotin-peroxidase immunostaining was performed by using the Histostain-SP (Zymed Laboratories, Burlingame, CA) kit. A 10-minute incubation of 3% H₂O₂ in methanol was applied to inhibit endogenous peroxidase activity, whereas a 20-minute incubation with 10% non-immune goat serum was used to reduce non-specific antibody binding. Then, the sections were incubated in a moist chamber at 48 °C overnight with the rabbit anti-androgen receptor antibody and 5 mg/mL PBS. Sections were then washed extensively with PBS containing 0.2% Triton X-100 and exposure to biotinylated goat anti-rabbit IgG for 30 min, followed by streptavidin-peroxidase for 30 min. The reaction was colored with a mixture of aminoethylcarbazole for 6 min. Light microscopy was employed in an analysis of five distinct areas of cells, subsequently stained. The number of strongly stained cells was ascertained and expressed as a percentage of all the stained cells.

2.11. Molecular docking study

To investigate the molecular impact of the administration of DEHP on testosterone levels, a molecular docking study was conducted to evaluate the affinity of testosterone and DEHP for androgen receptor and 5 α -reductase using finasteride as a 5 α -reductase inhibitor. A crystal structure of 5 α -reductase (PDB ID: 7BW1) and androgen receptor (PDB ID: 1t7f) was retrieved from the Protein Data Bank (<http://www.rcsb.org>). The three compounds testosterone (CID: 57363), DEHP (CID: 8343) and finasteride (CID: 57363) were retrieved from the PubChem database and converted to Protein Data Bank (PDB) format using Discovery Studio Visualizer. The structures were prepared and saved in PDBQT format, as described in a previous study[21]. To investigate the effects of DEHP on sex hormones, oxidative stress, and more, Gasteiger-type charges were assigned to the nonpolar hydrogens and then merged with the carbons. The internal degrees of freedom and torsions were also set and the ligands were converted into PDBQT format using Autodock tools. Binding affinities between testosterone, DEHP, and finasteride with two receptors, androgen receptor and 5 α -reductase, were determined using Vina[33] as previously described[21]. A detailed analysis of the binding affinities between the compound DEHP and androgen receptor and 5 α -reductase was conducted in order to understand the impact of DEHP on sex hormones. The analysis was done using Discovery Studio Visualizer in BIOVIA, 2016.

2.12. Statistical analysis

Data were analyzed using biostatistics software, Graph pad prism version 8.0 (Graph pad Software, Inc., Lajolla, USA). All data were presented as mean $\pm$ standard deviation (mean $\pm$ SD). Thereafter, analysis was carried out by one-way analysis of variance (ANOVA) followed by *post hoc* test (Tukey's) for multiple comparisons. For all tests, the level of significance was set at $P < 0.05$.

2.13. Ethics statement

This study was done under the approval of the Research, Ethics and Grants Committee of the Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria (REC/FBMS/DELSU/18/05). Experimental procedures were carried out with caution and in compliance with the NIH Guidelines for the care and use of laboratory animals.

3. Results

3.1. Effects of treatment with ZnSO₄ and NAC on gonadotropins and steroidal hormones in DEHP-induced reproductive toxicity in male Wistar rats

Figure 1 shows the effects of ZnSO₄ and NAC treatment on gonadotropins and steroidal hormones in male Wistar rats that were exposed to DEHP. Compared to the control group, the DEHP-exposed group had a statistically significant decrease in serum testosterone, oestrogen, LH and FSH ($P<0.05$). The results indicated that there was a statistically significant rise of testosterone and oestrogen in the groups given ZnSO₄, NAC and ZnSO₄+NAC, when compared to the DEHP-treated group ($P<0.05$). The ZnSO₄+NAC treated group also revealed a significant boost in serum LH, FSH

and testosterone, compared to the ZnSO₄ and NAC-treated groups, respectively ($P<0.05$).

3.2. Effects of treatment with ZnSO₄ and NAC on hormonal fertility index in DEHP-induced reproductive toxicity in male Wistar rats

In Figure 2, rats exposed with DEHP had a significant negative effect on the Leydig and Sertoli cell functions as evidence by decreased in hormonal fertility index [testosterone/LH (2A) and testosterone/oestrogen (2B)], respectively, when compared to the control groups. Notably, Figure 2 illustrates that the hormonal fertility index were enhanced in the groups that were treated with ZnSO₄, NAC, and ZnSO₄+NAC when compared to the DEHP-treated group.

3.3. Effect of NAC and ZnSO₄ on DEHP-induced alteration in kisspeptin–GnRH pathway in male Wistar rats

NAC and ZnSO₄ were investigated to assess their potential to mitigate DEHP-induced alterations to the kisspeptin–GnRH pathway. Specifically, serum levels of GnRH (Figure 3A) and Kisspeptin (Figure 3B) were measured in rats exposed to DEHP. Results showed that DEHP significantly reduced serum GnRH and kisspeptin levels when compared to the control group ($P<0.05$). An analysis of GnRH

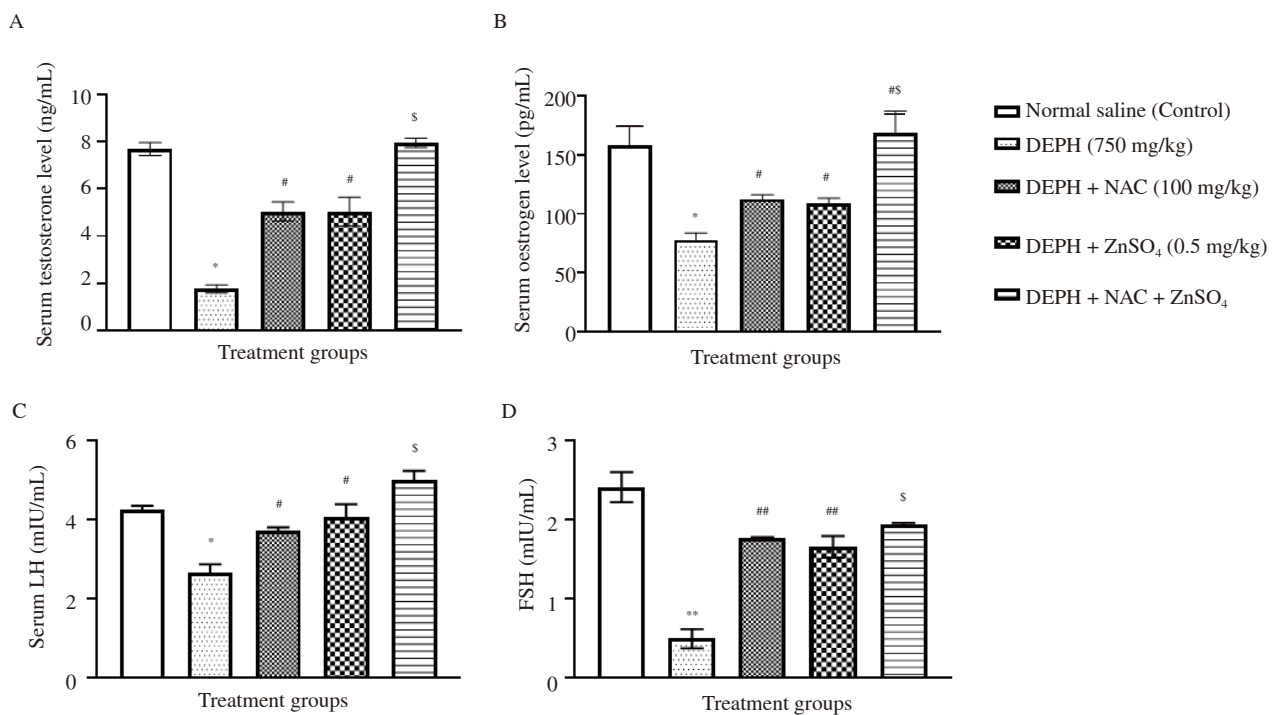


Figure 1. Effect of treatment with zinc sulphate (ZnSO₄) and *N*-acetylcysteine (NAC) on serum testosterone (A), oestrogen (B), luteinizing hormone (C) and follicle stimulating hormone (D) levels in DEHP-induced reproductive toxicity in male Wistar rats. Values are expressed as mean±SD ($n=7$) (one-way ANOVA followed by Tukey's *post hoc* test). * $P<0.05$, ** $P<0.01$: compared to the control group; # $P<0.05$, ## $P<0.001$: compared to the DEHP group; ^s $P<0.05$: compared to the NAC treated group. DEHP: Di-2-ethylhexyl phthalate, LH: luteinizing hormone, FSH: follicle stimulating hormone.

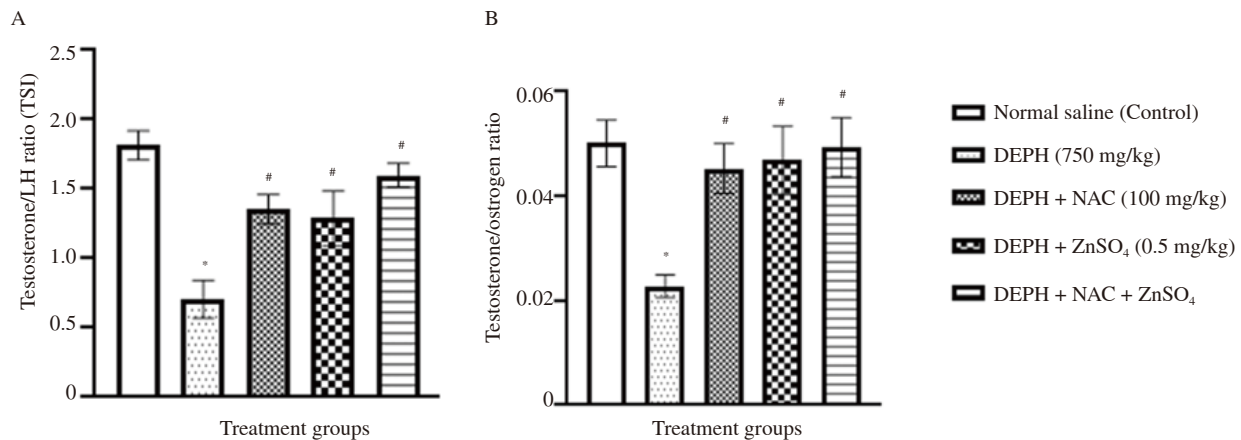


Figure 2. Effect of treatment with zinc sulphate (ZnSO₄) and *N*-acetylcysteine (NAC) on hormonal fertility indices in DEHP-induced reproductive toxicity in male Wistar rats: testosterone/LH (A) and testosterone/oestrogen (B). Values are expressed as mean±SD ($n=7$) (one-way ANOVA followed by Tukey's *post hoc* test). * $P<0.05$: compared to the control group; # $P<0.05$: compared to the DEHP group. DEHP: Di-2-ethylhexyl phthalate, LH: luteinizing hormone.

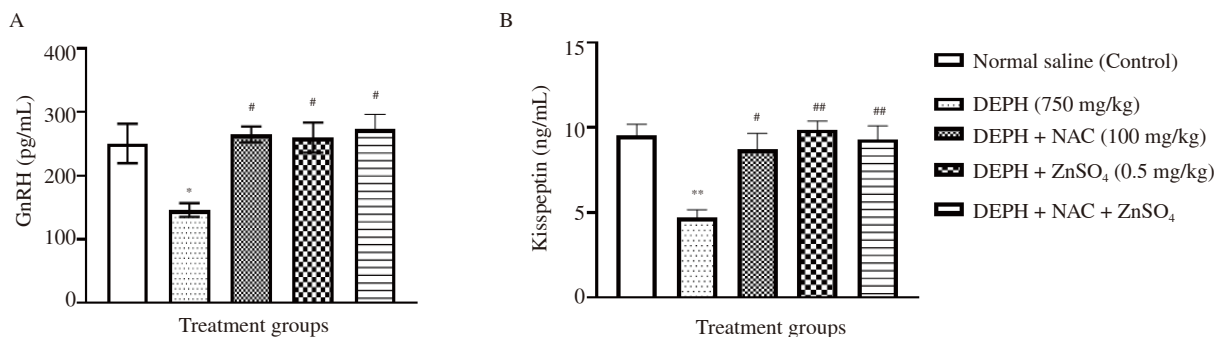


Figure 3. Protective effect of zinc sulphate (ZnSO₄) and *N*-acetylcysteine (NAC) on serum GnRH level in DEHP treated male Wistar rats. Values are expressed as mean±SD ($n=7$) (one-way ANOVA followed by Tukey's *post hoc* test). * $P<0.05$, ** $P<0.01$: compared to the control group; # $P<0.05$, ## $P<0.01$: compared to the DEHP group. DEHP: Di-2-ethylhexyl phthalate, LH: luteinizing hormone.

and kisspeptin levels in rats indicated that DEHP treatment caused a significant decrease in both hormones ($P<0.05$). However, the administration of NAC or ZnSO₄, or a combination of both, was shown to effectively mitigate the DEHP-induced negative alterations in these hormone levels ($P<0.05$). In particular, it was demonstrated that DEHP treatment led to a significant caused decline in serum kisspeptin levels compared to the control groups, but this decrease was reversed when NAC and/or zinc sulfate were given to the rats.

3.4. Effects of treatment with ZnSO₄ and NAC on DEHP-induced testicular oxidative stress in male Wistar rats

The effect of ZnSO₄ and NAC on testicular oxidative stress biomarker (MDA) and testicular antioxidant enzymes (SOD, catalase, GPx) in phthalate-induced reproductive toxicity in male Wistar rats is shown in Figure 4. Treatment with DEHP (750 mg/kg) produced a significant decrease in testicular SOD, catalase and GPx when compared with the control group ($P<0.05$). Treatment with DEHP significantly increased testicular malondialdehyde (MDA) level when compared with the control group ($P<0.05$). However, these alterations were profoundly decreased significantly by ZnSO₄,

NAC and ZnSO₄+NAC respectively when compared to DEHP group treated group alone.

3.5. Effects of treatment with ZnSO₄ and NAC on DEHP-induced testicular and pituitary gland histomorphological degeneration in male Wistar rats

The histomorphology of the testis (H & E, $\times 100$ magnification) from the DEHP treated group (Figure 5B) shows a very poor testicular architecture with the tubules lined by distorted spermatogenic series cells containing distended and congested vessels, some of the interstitial spaces and Leydig cells appears degenerated and congested when compared to the control group (Figure 5A). Testicular section from ZnSO₄ and NAC treated groups (Figure 5C and D) respectively showed tubules lined by spermatogenic series cells with numerous luminal spermatozoa. Congested vessels with aggregates of red blood cells were also seen. Histological sections of testicular tissue from NAC+ZnSO₄-treated group (Figure 5E) showed tubules lined by spermatogenic series cells and containing numerous luminal spermatozoa with no abnormality.

Figure 6 shows a pituitary sections in male Wistar rats. The control

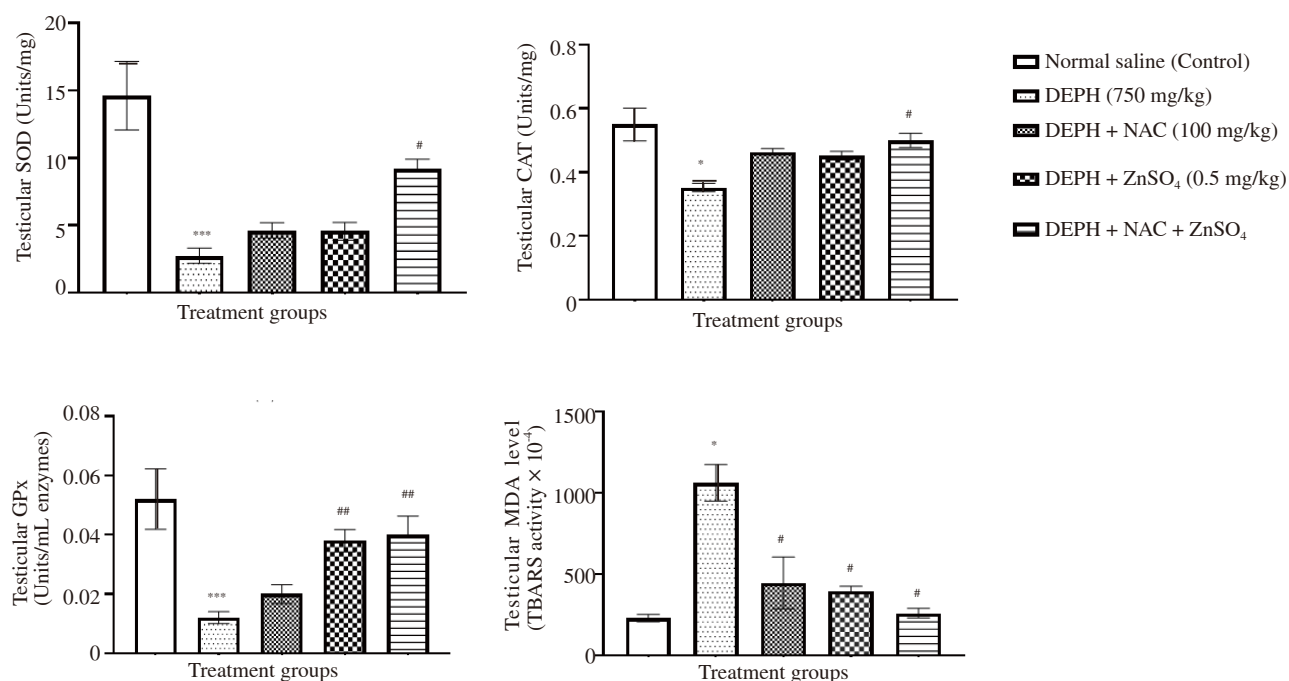


Figure 4. Effect of treatment with zinc sulfate (ZnSO₄) and *N*-acetylcysteine (NAC) on lipid peroxidation and anti-oxidant enzymes in DEHP-induced reproductive toxicity in male Wistar rats. Values are expressed as mean±SD (*n*=7) (one-way ANOVA followed by Tukey's *post hoc* test). **P*<0.05, ****P*<0.05: compared to the control group; #*P*<0.05, ##*P*<0.05: compared to the DEHP group. DEHP: Di-2-ethylhexyl phthalate, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, MDA: malondialdehyde.

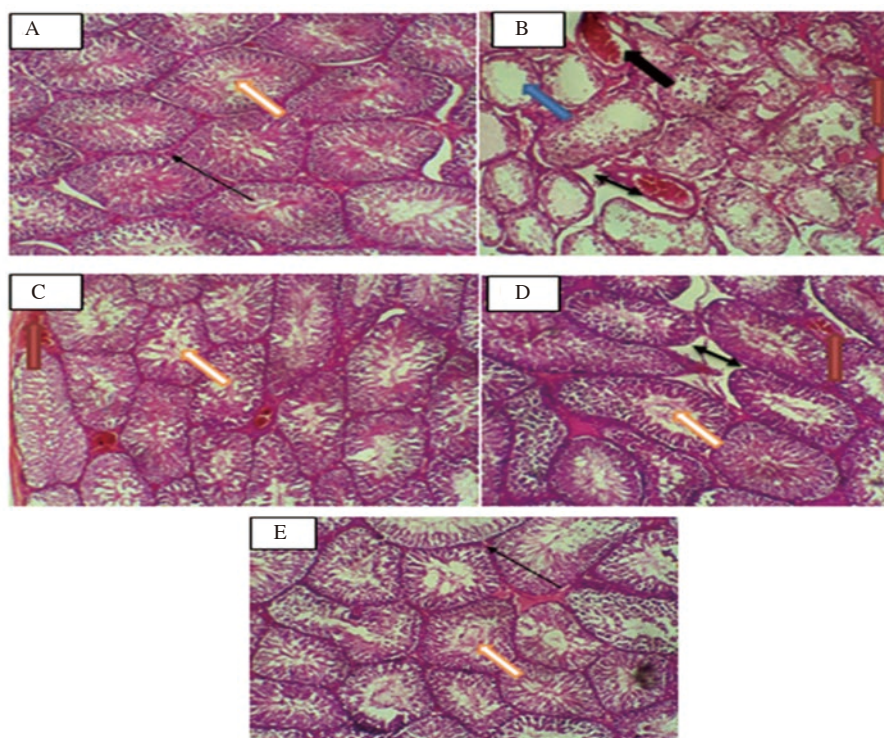


Figure 5. Effect of treatment with phthalate on the histology of the testis in male Wistar (H & E stain, ×100 magnification). A: Photomicrograph of testicular sections from the control group showing normal seminiferous tubules with normal germ cell layer with normal maturation stages, the lumen appear normal with presence of spermatozoa (white arrow). The interstitial spaces and Leydig cells appear normal (slender arrow). B: Photomicrograph of a testicular section from DEHP-treated group showing a very poor testicular architecture. The tubules are lined by distorted spermatogenic series cells and containing distended and congested vessels with aggregates of red blood cells (blue arrow). Some of the interstitial spaces and Leydig cells appear degenerated (spaned arrow) with congestion (orange arrows). C: Photomicrograph of a testicular section from ZnSO₄-treated group showing tubules lined by few distorted spermatogenic series cells and containing distended and congested vessels with aggregates of red blood cells indicating vascular congestion (orange arrow). D: testicular section from NAC-treated group showing tubules lined by spermatogenic series cells with numerous luminal spermatozoa (white arrow). Congested vessels with aggregates of red blood cells are also seen (orange arrow). E: Histologic sections of testicular tissue showing tubules lined by spermatogenic series cells and containing numerous luminal spermatozoa (white arrow). No abnormalities are seen.

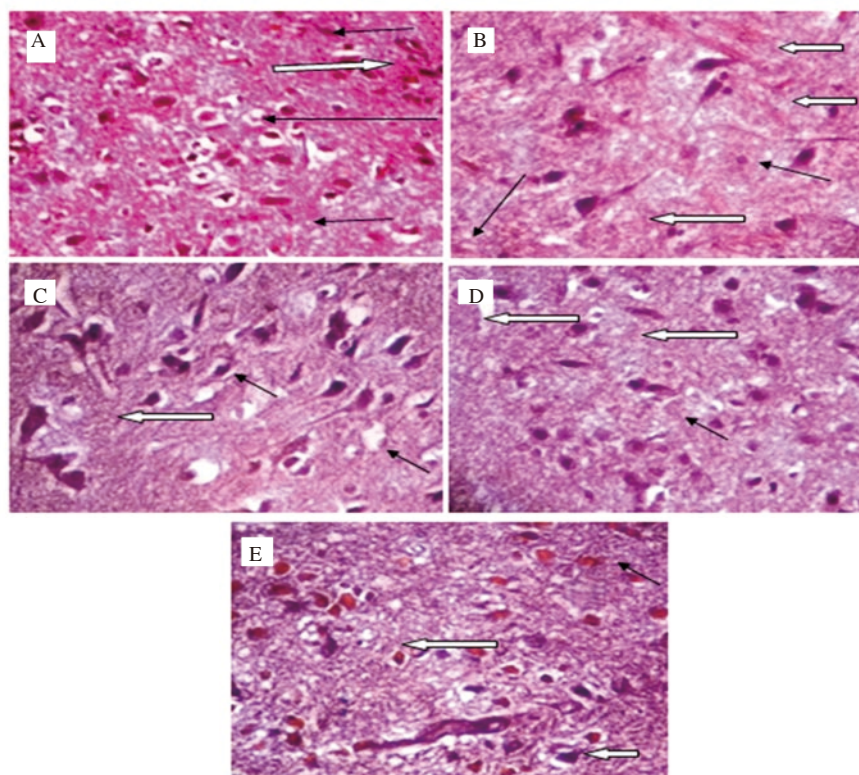


Figure 6. Effect of treatment with phthalate on the histology of the pituitary in male Wistar (PAS and orange G stains, $\times 400$ magnification). A: Photomicrographs of pituitary sections from the control group showing normal acidophilic cells and redish Basophills stained by PAS, beta cells appear red (white arrow), alpha cells appear orange (slender arrow). B: Photomicrograph of pituitary section from DEHP-treated rats showing depleted alpha cells (slender arrow) and depleted and few beta cells which appear (white arrow). C: Photomicrograph of pituitary section from a ZnSO_4 -treated rat showing depleted alpha cells (slender arrow) and moderate beta cells (white arrow). D: Photomicrograph of pituitary section from rats treated with NAC showing depleted alpha cells (slender arrow) and moderate beta cells (white arrow). E: Photomicrograph of section of pituitary from the group treated with combination of zinc sulphate and *N*-acetylcysteine showing moderately normal alpha cells (slender arrow) and moderate beta cells (white arrow).

group (Figure 6A) showed normal acidophilic (alpha) cells and basophills (beta) cells. Pituitary of DEHP-treated rat (Figure 6B) showed depleted alpha cells and very few beta cells. DEHP+ ZnSO_4 treated rat (Figure 6C) showed depleted alpha cells and moderate beta cells. DEHP+NAC treated rat (Figure 6D) displayed depleted alpha cells and moderate beta cells. DEHP+NAC+ ZnSO_4 treated rats (Figure 6E) showed moderately normal alpha cells and moderate beta cells.

3.6. Effects of treatment with ZnSO_4 and NAC on testicular androgen receptor expression in DEHP-exposed male Wistar rats

The photomicrograph from the immunohistochemistry assay showed upregulation of the testicular androgen receptor in the DEHP-treated group when compared with the control group. Testicular androgen receptor also showed a strong expression in the control group and a very strong expression in ZnSO_4 -treated group. The groups treated with NAC and ZnSO_4 +NAC showed a highly abundant expression of testicular androgen receptor (Figure 7).

3.7. Binding affinity of DEHP and testosterone on testicular androgen receptor and 5α -reductase

From the *in silico* study done to evaluate the binding affinities of the ligands for the target proteins to gain an insight into the cause of upregulation of the testicular androgen receptors, the result showed a very close binding affinity between testosterone (-12.6 Kcal/mol) and DEHP (-12.5 Kcal/mol) for the testicular androgen receptor, while for 5α -reductase, DEHP has a higher affinity (-14.0 Kcal/mol) when compared to that of testosterone (-12.7 Kcal/mol) but slightly lower than that of finasteride (-14.5 Kcal/mol) which was a standard inhibitor of the 5α -reductase.

3.8. Amino acid interaction of DEHP and testosterone on testicular androgen receptor

The amino acid interactions of testosterone and phthalate on the androgen receptor are shown in Figure 8A and Figure 8B, respectively. Figure 8A reveals that testosterone exhibited hydrogen bond interactions (green bonds) with PHE764 and MET749, while

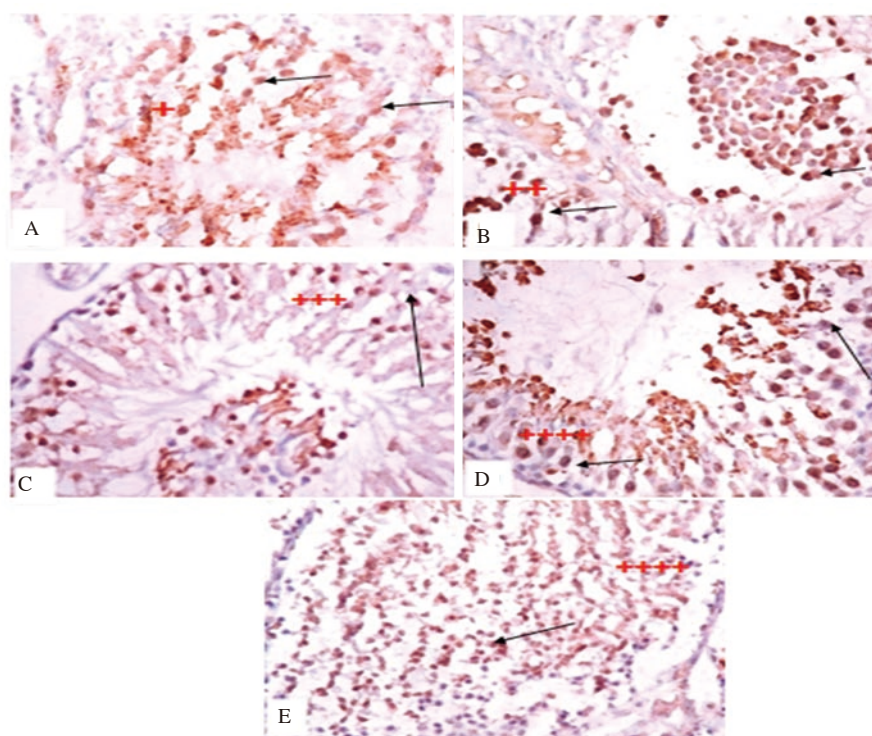


Figure 7. Photomicrograph of immunohistochemistry assay showing the effect of treatment with zinc sulphate and *N*-acetylcysteine on the quality of testicular androgen receptor in DEHP-exposed male Wistar rats ($\times 400$ magnification). (+) strong expression (53%), (++) very strong expression (60%), (+++) abundant expression (75%), (++++ highly abundant expression (greater than 90%), slender arrow (androgen receptor). A: Control, B: PHT treated, C: PHT+ZnSO₄ treated, D: PHT+NAC treated, E: PHT+ ZnSO₄+NAC treated group.

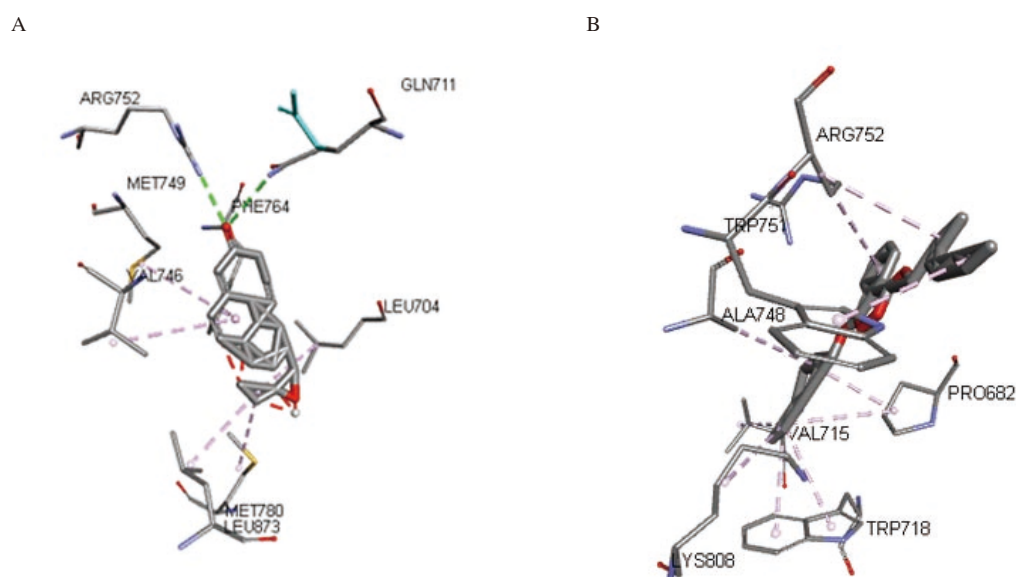


Figure 8. Interaction between amino acids on the binding site of androgen receptor and testosterone (A) and Di-2-ethylhexyl phthalate (DEHP) (B). A: Testosterone exhibiting hydrogen bond interactions (green bonds) with PHE764 and MET749, while exhibiting hydrophobic bond interactions with VAL746, MET780, LEU704 and ARG752; B: Phthalate interaction forming hydrophobic bonds with ARG752, TRP751, ALA748, PRO682, VAL715, LYS808 and TRP718. ARG: arginine, TRP: tryptophan, VAL: valine, ALA: alanine, PRO: proline, MET: methionine, LEU: leucine, LYS: lysine.

exhibiting hydrophobic bond interactions with LEU704, VAL746, ARG752, MET780 and LEU873. Figure 8B reveals that DEHP formed hydrophobic bonds with PRO682, VAL715, TRP718, ALA748, TRP751, ARG752 and LYS808.

3.9. Binding affinity and amino acid interaction of ligands with FSH receptor, LH receptor, and GnRH receptor

From the docking studies, Suramin, the standard FSH receptor antagonist, exhibited a binding energy of -12.4 Kcal/mol, relative to

-5.3 Kcal/mol for DEHP and -6.7 Kcal/mol for NAC. For LH and GnRH receptors, Abarelix exhibited a binding energy of -14.3 Kcal/mol and -14.1 Kcal/mol, respectively, compared to -5.9 Kcal/mol and -6.6 Kcal/mol by DEHP and -8.3 Kcal/mol and -8.6 Kcal/mol by NAC. ZnSO₄ exhibited a binding energy of -1.3 for FSH and GnRH receptors, and -1.1 for LH receptor. Hydrogen bond persists as the major form interaction, with other forms of hydrophobic interaction.

4. Discussion

This research has evaluated the potential of NAC and ZnSO₄, either alone or in combination, to mitigate reproductive toxicity caused by exposure to DEHP in male Wistar rats. Various end point markers were utilized, including serum levels of testosterone, LH, and estrogen, as well as antioxidant enzyme activity (SOD, catalase, and GPx), testicular and pituitary architecture, and testicular androgen receptor expression. Additionally, the binding abilities of testosterone, NAC, and ZnSO₄ for androgen receptor and 5 α -reductase were examined through in silico molecular docking, with finasteride serving as the standard 5 α -reductase inhibitor. Treatment with DEHP led to a decrease in serum testosterone, estrogen, and LH levels. This decrease in LH levels suggests that DEHP may harm pituitary function, potentially compromising the action of LH on Leydig cells and leading to decreased testosterone production. Furthermore, the decline in serum testosterone levels may be attributed to DEHP's direct inhibition of Leydig cell steroidogenesis and reduction in LH levels in the experimental subjects.

Moreover, multiple investigations have revealed that the majority of endocrine disruptors hinder the quality of sperm by decreasing the production of testicular LH, which is crucial for the production of androgen[26]. Various studies have previously demonstrated the detrimental effects of DEHP on Leydig cells. For instance, exposure to maternal DEHP has been shown to alter the steroidogenesis of Leydig cells in offspring[34,35], and phthalate therapy has been found to impact sperm parameters in a dose-dependent manner[36]. In experimental animals, the co-administration of DEHP with NAC and zinc sulphate has been proven to restore serum LH and testosterone levels, indicating the protective capabilities of NAC and ZnSO₄ against reproductive endocrine impairment caused by DEHP. While zinc has been previously linked to similar positive effects[1], the combination of ZnSO₄ and NAC has been associated with such activity. To confirm the impact of the treatment on reproductive hormonal function, hormonal fertility indices were also evaluated. These indices are considered better indicators of fertility than individual hormonal levels[37]. The testosterone/LH ratio is an indicator of Leydig cell function, while the testosterone/oestrogen ratio reflects aromatase enzyme activity and the function of germ cells and Sertoli cells. The study demonstrated a significant improvement in the reversal of these hormonal fertility indices in the group treated with a combination therapy of NAC and ZnSO₄

after exposure to DEHP. This suggests that the combination therapy has the potential to mitigate the adverse effects of DEHP on these indices. The discovery aligns with previous research that demonstrated the positive impact of zinc[36] and NAC[38,39] on reproductive functions in rodents, indicating their protective properties.

In order to assess the degradation of testosterone, the levels of oestrogen in the serum were also examined as the balance between these hormones is crucial in males. It has been observed that a decrease in testosterone levels leads to an increase in oestrogen levels, indicating the conversion of testosterone by 5 α -reductase or its binding to the androgen receptor. In the current study, a decrease in oestrogen levels was seen alongside the decrease in testosterone levels, suggesting that the conversion of testosterone may have been hindered by treatment with DEHP. However, the combined treatment of NAC and ZnSO₄ was able to mitigate the negative effects of DEHP on testosterone, possibly due to the antioxidant properties of these compounds. The administration of DEHP also had a detrimental impact on the structure of the testes, as observed through testicular sections which showed distorted tubules lined with spermatogenic cells containing congested and distended vessels, as well as aggregates of red blood cells and degenerated Leydig cells in the interstitial spaces. However, these harmful effects of the toxicant were alleviated by the co-administration of ZnSO₄ and NAC, either alone or in combination. The testes are protected by a tough membrane called the tunica albuginea, and within them are very fine coiled tubes known as seminiferous tubules. These tubules are lined with germ cells that mature from puberty to old age into sperm cells. The interstitial cells of Leydig, which are responsible for steroid production, are located between the seminiferous tubules, and any alteration in the normal structure of the testes can have detrimental effects on its function. This alteration of testicular structures is one mechanism by which toxic agents disrupt the functions of the testes. In light of the findings, it was determined that DEHP administration may have caused changes in the structure of the testes, potentially by decreasing the amount of functioning Leydig cells responsible for producing testosterone. This could explain the decrease in testosterone levels observed in this study after DEHP treatment. However, it was discovered that the combined treatment of ZnSO₄+NAC was able to reverse the negative effects of DEHP on testicular architecture, suggesting that these compounds may have the ability to restore the damage caused by DEHP.

Moreover, the study found that DEHP led to a decrease in both alpha and beta cells of the anterior pituitary gland. These beta cells are responsible for releasing LH into the bloodstream. The decrease in beta cells may have caused the observed drop in serum LH levels and subsequent decrease in testosterone, potentially due to DEHP's pro-oxidative properties. This suggests that one of the ways in which DEHP affects testosterone levels is through depleting pituitary cells. However, this mechanism was prevented by the co-administration of ZnSO₄ and NAC, further supporting the idea that these agents

may be beneficial in mitigating reproductive toxicity caused by chemicals.

The observed increase in androgen receptor levels after exposure to DEHP may be a response to the decrease in serum testosterone, in order to maximize the reduced amount of testosterone available. Co-treatments with ZnSO₄ and NAC, either separately or in combination, were found to further upregulate the androgen receptor. This implies that even with lower levels of testosterone, there is a significant improvement in testicular function. The elevated expression of androgen receptor seen in the therapeutic intervention may also be attributed to the potential impact of DEHP on energy levels. A previous study by Rowdhwal and Chen[2] suggested that high phthalate levels could lead to a deficit in testicular energy due to inhibition of adenosine triphosphate (ATP) production. Furthermore, recent research has shown that supplementation of testosterone can enhance androgen receptor expression even under conditions of extreme energy deficiency. Additionally, there is no evidence to suggest that NAC can provide energy to the body or improve energy status during times of stress, and there are no reports linking zinc to energy balance. Hence, the increased expression observed in the therapeutic intervention may be an adaptive response to the potential energy deficit caused by DEHP, as previously reported[2].

Sperm cells contain high levels of PUFAs, making them susceptible to oxidative stress which can lead to loss of testicular enzymes and inhibition of spermatogenesis. Studies have shown a link between increased production of reactive oxygen and nitrogen species (ROS/RNS) and altered spermatogenesis and decline in reproductive hormones. Previous research has also demonstrated that exposure to DEHP can cause reproductive toxicity through increased production of ROS/RNS, resulting in depletion of PUFAs in cell membranes. In order to protect cells from the damaging effects of ROS, such as superoxide anion, hydroxyl radical, and peroxides[40–42], antioxidant defense mechanisms are essential. Results from this current study indicate that rats treated with DEHP had elevated levels of MDA and decreased levels of antioxidant enzymes (SOD, catalase, and GPx). However, co-treatment with NAC and ZnSO₄ resulted in a decrease in MDA levels and an increase in antioxidant enzyme activity, suggesting that NAC and ZnSO₄ have anti-lipid peroxidative and antioxidant properties that can suppress oxidative damage in treated animals. To better understand why levels of testosterone decrease after treating animals with DEHP, the molecular interactions of DEHP and testosterone with androgen receptor and 5 α -reductase were investigated through *in silico* molecular docking, using finasteride as a reference 5 α -reductase inhibitor[21]. Androgen receptor is a nuclear receptor that is activated by androgenic hormones, including testosterone, while 5 α -reductase converts testosterone to dihydrotestosterone. The level of testosterone is greatly influenced by the binding of administered compounds to these two protein targets. The *in silico* study revealed that DEHP, finasteride, and testosterone have binding energies of -14.0 Kcal/mol, -14.5 Kcal/mol, and -12.7 Kcal/mol for 5 α -reductase, respectively.

A more negative binding energy indicates a stronger binding affinity. The results showed that DEHP had a higher binding affinity (-14.0 Kcal/mol) for 5 α -reductase compared to testosterone (-12.7 Kcal/mol). This aligns with a previous study by Beg and Sheikh[43]. The binding energy patterns of DEHP and testosterone on androgen receptor and 5 α -reductase indicate the potential for DEHP to disrupt androgen receptor signaling, which could lead to reproductive dysfunction related to androgens. Nevertheless, the observed decline in testosterone levels may not be a result of its conversion to dihydrotestosterone by 5 α -reductase. This is because DEHP is likely to inhibit the enzyme, as evidenced by its higher binding affinity compared to testosterone. Additionally, while testosterone's binding affinity to androgen receptor at -12.6 Kcal/mol may not be significantly affected by DEHP, it is worth noting that both DEHP and testosterone may share a common binding site at position 752 on the androgen receptor amino acid sequence. These findings suggest that the decrease in testosterone levels caused by DEHP in the *in vivo* study may not be due to DEHP competing with testosterone for the androgen receptor binding site or binding to 5 α -reductase. Instead, it may be attributed to DEHP's toxic effects on the beta LH secreting cells of the anterior pituitary and the Leydig cells of the testes, which could impact testosterone production. However, the binding energy pattern of DEHP and testosterone with androgen receptor also indicates the potential for DEHP to disrupt androgen receptor signaling and lead to androgen-related reproductive dysfunction. Thus, further studies are needed to fully characterize the mechanisms by which NAC and ZnSO₄ may counteract the toxic effects of DEHP on the male reproductive system.

The highest binding affinity was observed for the verified antagonists of the receptor, with more negative binding energy being associated with higher binding affinity[41,42]. Additionally, the results indicated that NAC had a higher binding energy for all receptors compared to phthalate. This could potentially explain why NAC is effective in protecting against the reproductive toxicity of phthalate when administered together. On the other hand, ZnSO₄ exhibited a low binding tendency with the receptors in comparison to all other ligands studied. However, the modulatory effect of Zn ion on these receptors cannot be solely attributed to its binding tendency, as Zn ion is a component of various enzymes (such as carboxypeptidase and carbonic anhydrase) and also acts as a cofactor for certain enzymes, including the antioxidant enzyme superoxide dismutase.

The potential limitation of this study is that the study was conducted on rats and not humans, so the results may not necessarily apply to humans. Further research on humans would be needed to confirm the findings.

In conclusion, the findings of this research showed that DEHP negatively affected the pituitary and gonadal functions in the animals that received treatment. However, when ZnSO₄ and NAC were given together, they were able to restore the normal functioning of the endocrine system and balance the redox status in the experimental rats. This was achieved through the inhibition of oxidative stress

and modulation of the pathways related to male sex hormones. It is possible that the reduction of testosterone disruption caused by DEHP by NAC and ZnSO₄ can be attributed to their ability to counteract reproductive toxicity.

Conflict of interest statement

Authors declare that they have no conflict of interest

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Data availability statement

Data are available on request from the corresponding author.

Authors' contributions

Eze Kingsley Nwangwa and Alexander Obidike Naiho conceived, designed and co-supervised the research; Motunrayo Lade-Ige and Victor Omo-Idonije conducted the experiments; Victor Oghenekparobo Emojevwe, Kayode Ezekiel Adewole, Peggy Ejio Ohwin, Adeniran Oluwadamilare Akinola and Benneth Ben-Azu wrote the manuscript and contributed reagents; Mega Obukohwo Oyovwi and Victor Oghenekparobo Emojevwe conducted experiments while Kayode Ezekiel Adewole did the insilico docking with Oghenekparobo Emojevwe. Mega Obukohwo Oyovwi collected samples and analyzed the data and helped Victor Oghenekparobo Emojevwe with literature search. All authors read and approved the manuscript.

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