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Toxicological outcome of phthalate exposure on male fertility: Ameliorative impacts of the co-administration of N-acetylcysteine and zinc sulfate in rats

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Abstract

Background: Reports have shown that humans are consistently exposed to environmental toxicants such as phthalate (PHT) during their daily activities. This results in reproductive dysfunction and infertility-related issues as already noted in human and experimental animals. We therefore designed this study to investigate fertility outcome in phthalate-exposed male rats treated with N-acetylcysteine (NAC) and zinc sulfate (ZnSO4) with the view of providing a therapeutic alternative to reproductive toxicity caused by phthalate. The research was done in two phases. In phase 1, thirty-five male Wistar rats were randomly assigned to one of five ($n = 7$) groups given the following treatments for 21 days: group A was given distilled water as a control, while groups B, C, D, and E were given phthalate (750 mg/kg/day). Animals in groups C to E were also given ZnSO4 (0.5 mg/kg/day), N-acetylcysteine (100 mg/kg/day), and ZnSO4 (0.5 mg/kg/day) + N-acetylcysteine (100 mg/kg/day) in addition to phthalate. In phase 2, animals from groups in phase 1 were mated with females for fecundity testing.

Results: The result shows alteration in testicular and epididymis weight and testis/epididymis ratio, semen parameters, sperm capacitation and acrosome reaction, sperm DNA, serum Zn and Mg, testicular mitochondria apoptosis mechanisms (TNF-α and BCL-2), and testicular *Ca^{2+}-ATPase* as well as fecundity outcome in the phthalate-treated group. However, $ZnSO_4$ and NAC successfully ameliorated the deleterious effects of phthalate on semen parameters, sperm capacitation and acrosome reaction, serum electrolyte and mitochondria apoptosis mechanisms, and testicular electrogenic *Ca^{2+}-ATPase* in phthalate-induced male rats with a better outcome in the combined therapy. Pregnancy outcome and litter sizes were also higher in the combined therapy when also compared with the phthalate-treated groups.

Conclusion: According to the result, $ZnSO_4$ and NAC increased fertility outcome in phthalate-treated male rats through enhancement of testicular BCL-2, serum electrolyte, testicular *Ca^{2+}ATPase* pumps, and cytoprotection.

Keywords: N-acetylcysteine, $ZnSO_4$, Semen parameters, Sperm capacitation and acrosome reaction, Chromatin integrity, Ca^{2+}ATPase, BCL-2, TNF-α

Background

According to reports, humans are routinely exposed to environmental toxins and endocrine-disrupting chemicals such as phthalate during normal human activities [1–3]. Phthalate is a synthetic substance that is used to provide flexibility and solubility in products such as

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medication coatings, blood and urine bags, infusion fluid bag, hand gloves, adults and children toys, cosmetics, and many other consumer products [4, 5]. Unfortunately, this chemical also acts as an endocrine disruptor and thereby causes reproductive dysfunction in human and experimental animals leading to fertility issues [6]. Moreover, an experimental evidence suggests that phthalates may have developmental and reproductive toxic effects, confirming their role in infertility [7].

Infertility is a problem associated with the reproductive system which prevents a couple from achieving pregnancy despite frequent, unprotected sexual intercourse for a year or more [8]. The American Pregnancy Association considers the condition to be a disease linked to disorder or termination of the functions, processes of organs of either the male or female reproductive tract that prevents the conception of a child [9]. It is estimated that 10–15% of all couples are affected [10–12], resulting in approximately 186 million cases of infertility worldwide, with male factors accounting for more than half of these cases [13]. Male infertility is on the rise in Nigeria and many other countries around the world [14, 15], emphasizing the importance of studying the effects of environmental toxins on male infertility.

The mechanism by which phthalate causes assaults on the male reproductive system is still being studied [16, 17], and various treatment approaches are still required for the management of phthalate-induced toxicity [18–20].

A previous study linked the effects of phthalate to oxidative stress [21], while others postulated that the male reproductive tract is highly susceptible to effects of oxidative stress [22, 23]. Consequently, it is believed that oxidative stress is likely to play a role in the adverse reproductive toxicity caused by phthalate administration [22, 24].

N-acetylcysteine (NAC), a widely used antioxidant, is a precursor to the amino acid L-cysteine and results in the antioxidant glutathione [25], while zinc sulfate, another antioxidant agent, has been implicated with DNA replication, RNA polymerases, protein synthesis, growth processes, and a variety of metabolic processes [26]. The present study was therefore conducted on the premise that well-established antioxidants such as N-acetylcysteine and zinc sulfate may mitigate changes in testicular functions caused by chronic phthalate exposure, thereby improving fertility outcome.

Methods

Experimental animal model

Sixty-five adult Wistar rats weighing between 150 and 200g (16–18 weeks old) were used in this experiment, thirty-five of which were males and thirty were virgin females. The animals were bred in the animal house unit of the same institution where the study was done and were kept under a standard laboratory condition with a 12:12-h light and dark cycle at 25°C ± 2°C and allowed free access to standard commercial rat pellets with standard composition and water ad libitum. The animals were acclimatized for 2 weeks prior to the start of drug administration.

Experimental design

After acclimation, the research was designed into two experimental phases. While phase 1 is an ameliorative investigation, phase 2 was designed for fecundity testing.

Phase 1 (ameliorative study)

This phase included thirty-five male Wistar rats randomly assigned to one of five groups ($n = 7$) and were treated for 3 weeks based on the results of previous studies. Group A which served as the control received distilled water as *placebo* for 21 days, group B served as the treated control and received phthalate (750 mg/kg/day) only for 21 days, group C received phthalate (750 mg/kg/day) + $ZnSO_4$ (0.5 mg/kg/day) for 21 days, group D received phthalate (750 mg/kg/day) + NAC (100 mg/kg/day) for 21 days, and group E received phthalate (750 mg/kg/day) + NAC (100 mg/kg/day) + $ZnSO_4$ (0.5 mg/kg/day) for 21 days. All the drugs were given via the oral route of drug administration.

Phase 2 (fecundity testing)

Forty adult Wistar rats which consisted of 10 males drawn from groups in phase 1 and 30 virgin females were randomized into five groups for the sake of mating. The animals were mated in separate cages by pairing 2 males from each treatment group in phase 2 with 6 virgin females as outlined below:

Group F = 6 females mated with 2 males from the control
Group G = 6 females mated with 2 males from the group treated with phthalate (750 mg/kg/day)
Group H = 6 females mated with 2 males from the group treated with phthalate (750 mg/kg/day) + $ZnSO_4$ (0.5 mg/kg/day)
Group I = 6 females mated with 2 males from the group treated with phthalate (750 mg/kg/day) + NAC (100 mg/kg/day)
Group J = 6 females mated with 2 males from the group treated with phthalate (750 mg/kg/day) + NAC (100 mg/kg/day) + $ZnSO_4$ (0.5 mg/kg/day)

Collection and administration of drugs

The zinc sulfate used in the study was obtained from Uche-care pharmaceutical shop in Ondo, while the phthalate and N-acetylcysteine were obtained from Sigma Aldrich, USA. The chemicals were given to the animals orally using the oro-gastric cannula. The animals received phthalate at a dosage of 750 mg/kg/day as modified from previous studies [6, 27] and N-acetylcysteine was given at a dose of 100 mg/kg as recommended in a previous study [14], while zinc sulfate was given at a dose of 0.5 mg/kg/day also according to a previous study by Nawal et al. [28].

Mating and confirmation of pregnancy

The process of mating and confirmation of pregnancy were done according to the method of Ochiogu et al. [29] also described by Devon [30]. After the female rats were distributed, a vaginal smear of each of them was made on a clean glass slide by carefully inserting a cotton buds swab moistened with normal saline into the rats' vaginal cavity. The swabs were gently rubbed against the vaginal wall and carefully rolled around before being removed. The moist swab was immediately smeared onto a labeled clean glass slide. The smear was examined under a light microscope to look for the presence of protein coagulate. Each rat was then labeled with an indelible marker of a different color. The male rats were then introduced into the cages and were allowed to stay with the females for 5 days during which observation was made every morning.

Sample collection

At the end of the experimental period in phase 1, the animals were fasted overnight and euthanized by light thiopentone sodium. Laparotomy was done and the blood sample was collected by cardiac puncture while the testes and epididymis were carefully harvested and weighed on an electronic weighing balance. The epididymis was used for semen analysis and the testes were homogenized for biochemical assays while the blood sample was centrifuged and serum was collected for electrolyte assay.

Semen analysis

The semen was analyzed by the *conventional* manual microscopic *methods as described under the subheadings below:*

Epididymal sperm motility

Sperm motility was determined by a conventional method of Khatun et al. [31]. After the sperm was milked on the pre-heated slide, two drops of 2.9% sodium citrate were added. This was then concealed by a cover slip and examined under a microscope using a low-light ×40 objective [31].

Epididymal sperm viability (live/death ratio)

This percentage of spermatozoa in a unidirectional progressive movement across a field on a slide was observed with a light microscope fitted with a camera using the eosin/nigrosin stain; the specimen used for epididymal sperm motility was retrieved and the cover slip was quickly removed and two drops of eosin/nigrosin stain were added and a smear was made, air-dried, and viewed under the light microscope [32]. Because of their intact cell membranes, living sperm cells were unstained, whereas dead sperm cells took up the stain (because of their damaged cell membrane). The percentage of live/dead was calculated by counting 100 cells as described in previous studies [31, 33].

Sperm morphology

On a clean slide, a thin coating of the sperm sample was applied, which was then fixed with 95% ethanol and air-dried. The slide was then sequentially immersed in different concentrations of ethanol followed by staining with Harris hematoxylin, G-6 orange stain, and EA-50 green stain for 1 min each. The slide was then microscopically examined at ×1000 magnification and 200 sperm were analyzed and sperm anomalies were expressed in percentages [32, 33].

Epididymal sperm count

This was done as described by Omirinde et al. [32]. The caudal portion of the epididymis was homogenized in formal saline, and sperm counting was performed using the enhanced Neubauer Chamber (LABART, Germany) under the light microscope at a magnification of ×40.

Sperm capacitation and acrosome reaction

Sperm samples were obtained by milking the caudal epididymis of rats into a pre-warm modified sperm capacitation medium (SCM) as described by Bailey [34]. To an Eppendorf tube containing 1ml of SCM, 100 µl of sperm sample was transferred and incubated in a damp atmosphere of 5% CO_2 for 3 h at 37°C. An aliquot of the sperm was removed from each group and sperm acrosome status was then estimated using the Coomassie brilliant blue staining technique [35]. On glass slides, sperm samples were air-dried and fixed with ethanol. After drying, the slides were submerged in a 5% solution of paraformaldehyde in PBS for 15 min and then washed once with PBS. The slides were stained with aqueous 0.25% CBB R-250 in 10% glacial acetic acid and 25 percentage methanol, rinsed with water, cleaned, air washed, and sealed with cover lips under mounting media (Olympus, Japan). The acrosome region was stained blue in the acrosome-intact sperm while the acrosome-reacted sperm were unstained [35]. Then, for capacitated sperm

cells, the head was stained. Each acrosome assessment represents 5 to 6 microscopic fields with 80 to 100 sperm in each field.

Assessment of sperm DNA damage using the toluidine blue staining technique

This was done as described by Selvam and Agarwal [36] in which a thin smear was prepared with the semen sample, air-dried, and fixed in 96% ethanol and acetic acid solution of equal ratio (1:1) for about 30 min at 4°C. The slides were treated with 0.1M HCl for 5 min at 4°C after which distilled water was used to wash them 3 times for 2 min and then stained with 0.05% toluidine blue stain for 10 min. The slides were then examined under the light microscope at a magnification of ×4 to observe the heads of the spermatozoa as established by [37].

Electrolyte (Zn and Mg) level determination

Electrolytes (Zn and Mg) were determined by the enzyme-based immunoassay (EIA) system by the help of the automated electrolyte analyzer described by Karen [38].

Testicular TNF-α and BCL-2 analysis

These parameters were measured using ELISA [39] after reagents, tests, and standards were prepared in accordance with the manufacturer's instructions.

Testicular tumor necrotic factor-alpha (TNF-α) analysis was done by using the tumor necrosis factor-alpha (TNF-α) kit for rat testicular homogenates according to the method described by Karna et al. 2019 [40]. Both reagents, tests, and standards were prepared in accordance with the manufacturer's instructions. One hundred microliters of standard or sample was added to each well and incubated for1 h at 37°C after which it was aspirated. One hundred microliters of prepared detection reagent A was added and incubated again for 1 h at 37°C, aspirated, and washed 3 times. One hundred microliters of prepared detection reagent B was applied, and it was incubated for 30 min at 37°C before being aspirated and washed 5 times. The 90-µl substrate solution was then applied, and the incubation time was increased to 10–20 min at 37°C. Finally, 50 µl of stop solution was applied, and the reading at 450nm was taken right away.

Testicular B-cell lymphoma-2 (BCL-2) analysis was also measured using the ELISA method, using the B-cell lymphoma-2 (BCL-2) kit designed for rat testicular homogenates according to the method of Adams et al. 2019 [39]. Accordingly, 100-mg testicular tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS, and stored overnight at −20°C. The sample was centrifuged again after thawing before the assay. All reagents and standards were prepared as described in the kit user's guide. One hundred microliters of standard and sample per well was added to the prepared reagent, covered with an adhesive strip provided, and incubated for 2 h at 37°C. The liquid of each well was then removed. One hundred microliters of Biotin-antibody (1x) was added to each well, covered with a new adhesive strip, and incubated for 1 h at 37°C. After incubation, each well was aspirated and washed and the process was repeated two times. The wells were then washed by filling each well with wash buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, and let it stand for 2 min. After the last wash, any remaining wash buffer was decanting after which the plate was inverted and blotted against clean paper towels. One hundred microliters of HRP-avidin (1x) was added to each well and the micro-titer plate was covered with a new adhesive strip and incubated for 1 h at 37°C. The aspiration/wash process was repeated for five times. Ninety microliters of TMB substrate was added to each well and incubated for 15–30 min at 37°C. Fifty microliters of stop solution was then added to each well and mixed thoroughly. The optical density of each well was determined within 5 min, using a microplate reader set to 450 nm.

Determination of Ca^{2+} ATPase activity in testicular homogenate

This was done based on a modification of the method described by Olaniyan et al. [41] in which 0.5 ml of each of 21.0 mM magnesium chloride, 17.5 mM calcium chloride, 10 mM of Tris HCl at PH 7.4, and 8.0 mM disodium ATP was mixed together in a test tube. 0.2 ml of tissue homogenate was added to it and incubated at 37 °C for 60 min. The reaction was brought to an end by adding 0.8 ml of ice-cold 10% (w/v) trichloroacetic acid (TCA). It was then allowed to stand at 4°C for 20 min and centrifuge at 4000 rpm for 5 min. To 1 ml of the supernatant was then added 1 ml of 1.25% ammonium molybdate and wait for 10 min. Then, 1 ml of 9% ascorbic acid was added and kept at room temperature for 20 min and the absorbance was measured at 725 nm using a spectrophotometer.

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 ± SEM (standard error of mean). 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 $p < 0.05$.

Results

Effects of treatment with zinc sulfate and N-acetylcysteine on organ weight in phthalate-treated male Wistar rats

Figure 1a shows a statistically significant decrease in testicular weight [F (4, 20) = 12.41, $p < 0.0001$] in the group exposed to the PHT (750 mg/kg)-treated group when compared with the control ($p < 0.05$). However, there was a statistically significant increase in testicular weight in PHT+$ZnSO_4$, PHT+NAC, and PHT+$ZnSO_4$+NAC groups when compared with the PHT (750 mg/kg) group. Figure 1b shows a similar statistically significant reduction in weight of the epididymis [F (4, 20) = 6.841, p = 0.0012] in PHT-treated rats when compared with control ($p < 0.05$). The result also shows an increase in weight of the epididymis in phthalate groups co-treated with PHT+$ZnSO_4$, PHT+NAC, and PHT+$ZnSO_4$+NAC when compared with the group treated with only PHT (750 mg/kg). Furthermore, Fig. 1c shows a statistically decrease in epididymis/testis ratio [F (4, 20) = 3.716, p = 0.0203] in the PHT-treated group when compared with the control ($p < 0.05$). It also shows a significant decrease in epididymis/testis ratio [F (4, 20) = 6.008, p = 0.0024] in PHT groups co-treated with $ZnSO_4$ (0.5 mg/kg) and $ZnSO_4$+NAC when compared with the group treated with only PHT (750 mg/kg), although there was a concurrent decrease in epididymis/testis ratio in the NAC (100 mg/kg) co-treated group but this change was not statistically significant when compared with the PHT-treated group.

Effects of treatment with zinc sulfate and N-acetylcysteine on semen parameters of phthalate-treated male Wistar rats

Figure 2A shows the effects of treatment with $ZnSO_4$ and N-acetylcysteine on sperm count in phthalate-treated male Wistar rats. Sperm count [F (4, 20) = 14.57, $p < 0.0001$] was significantly reduced in the PHT-treated group when compared to the control group. However, Fig. 2A also shows a significantly higher sperm count in the PHT+$ZnSO_4$+NAC-treated group when compared with the PHT-treated group as well as the PHT+$ZnSO_4$- and PHT+NAC-treated groups respectively. Figure 2B shows the effects of treatment with $ZnSO_4$ and NAC on sperm motility [F (4, 20) = 24.21, $p < 0.0001$] in phthalate-treated male Wistar rats. Accordingly, sperm

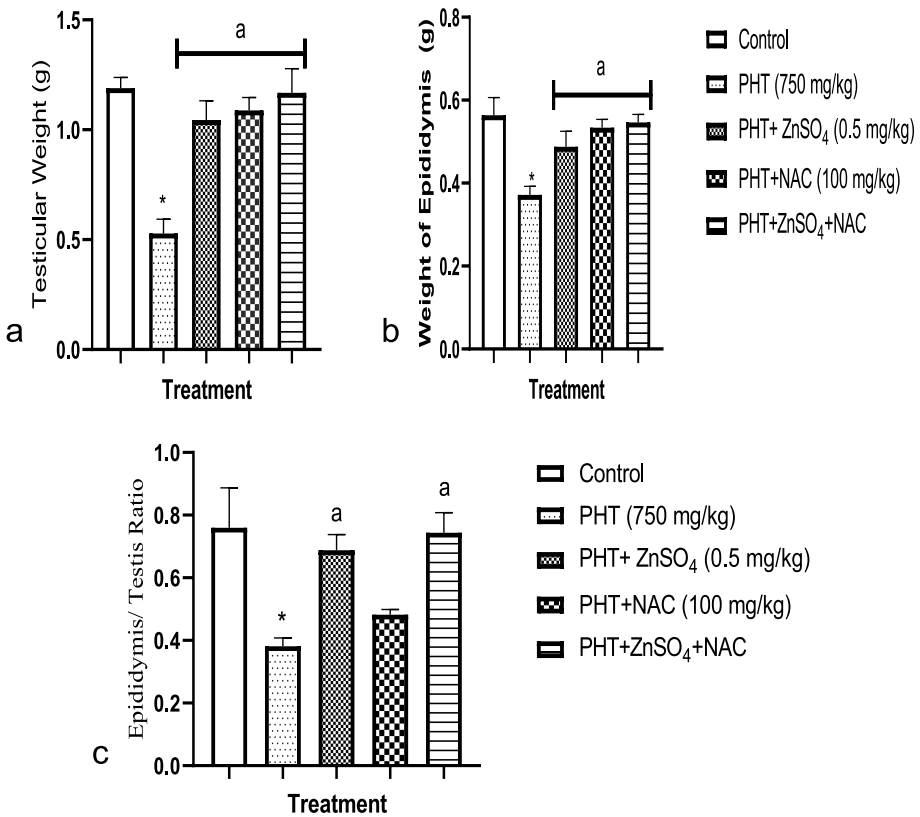

Fig. 1 Effects of treatment with zinc sulfate and N-acetylcysteine on organ weight in phthalate-treated male Wistar rats. Values are expressed as mean ± SEM ($n = 5$) (one-way ANOVA followed by Tukey's post hoc test). PHT phthalate, $ZnSO_4$ zinc sulfate, NAC N-acetylcysteine. * and a $p < 0.05$ were considered statistically significant when compared with the control and PHT-treated groups respectively

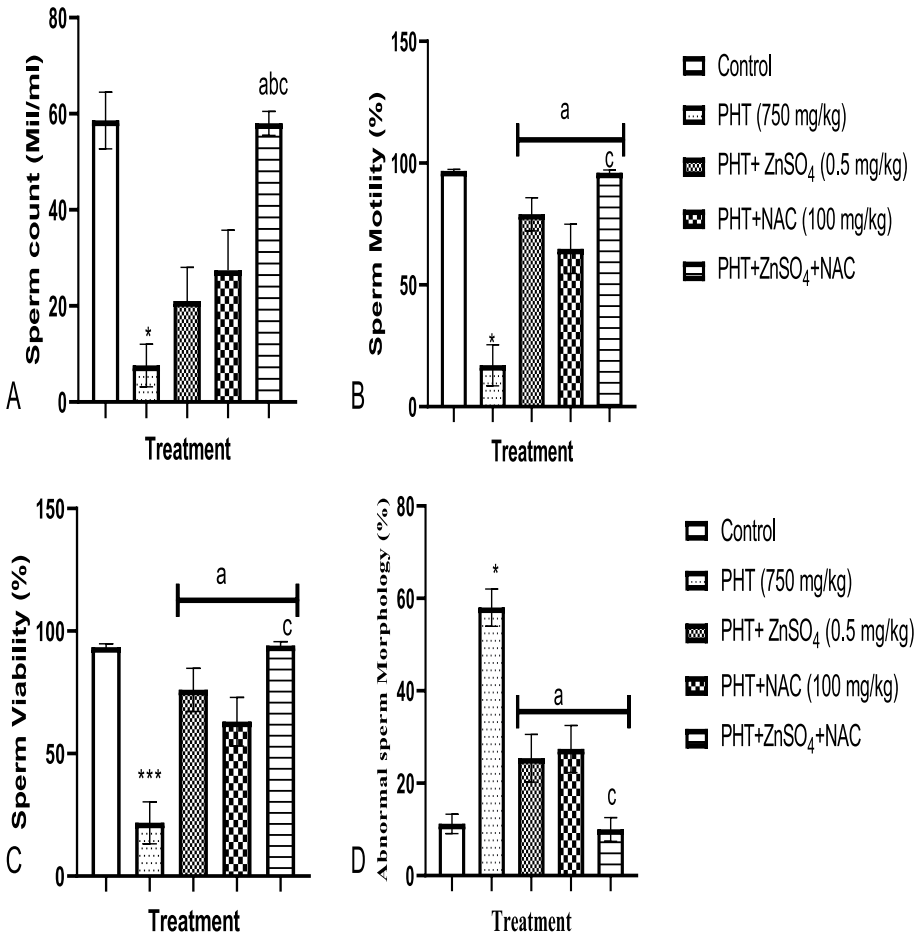

Fig. 2 Effects of treatment with zinc sulfate and N-acetylcysteine on semen parameters of phthalate-treated male Wistar rats. Values are expressed as mean ± SEM ($n = 5$) (one-way ANOVA followed by *Tukey's post hoc test*). PHT phthalate, $ZnSO_4$ zinc sulfate, NAC N-acetylcysteine. *, ***$p < 0.05$ and $p < 0.001$ were considered statistically significant when compared with the control, while **a**, **b**, and **c** $p < 0.05$ were statistically significant when compared with PHT-, PHT+$ZnSO_4$-, and PHT+NAC-treated groups respectively

motility was significantly reduced in the PHT-treated group when compared to the control group while it was concurrently higher in the PHT+$ZnSO_4$-, PHT+NAC-, and PHT+$ZnSO_4$+NAC-treated groups when compared with the PHT-treated group respectively. The higher sperm motility in PHT+$ZnSO_4$+NAC-treated is significant when compared to the PHT+NAC-treated group. Figure 2C shows the effects of treatment with $ZnSO_4$ and NAC on sperm viability [F (4, 20) = 17.26, $p < 0.0001$] in phthalate-treated male Wistar rats. Accordingly, sperm viability significantly reduced in the PHT-treated group when compared to the control group ($p < 0.05$). However, there was also increased sperm viability in PHT+$ZnSO_4$-, PHT+NAC-, and PHT+$ZnSO_4$+NAC-treated groups when compared with the PHT-only-treated group respectively with a better outcome in the PHT+$ZnSO_4$+NAC-treated group. The result in Fig. 2D shows the effects of treatment with $ZnSO_4$ and NAC on sperm morphology in phthalate-treated male Wistar rats. Percentage of spermatozoa with abnormal morphology significantly increased [F (4, 20) = 23.34, $p < 0.0001$] in the PHT-treated group when compared with the control while treatment with PHT+$ZnSO_4$, PHT+NAC, and PHT+$ZnSO_4$+NAC showed reduced numbers of spermatozoa with abnormal morphology with a better outcome in the PHT+$ZnSO_4$+NAC-treated group (Fig. 2D).

Effects of treatment with zinc sulfate and N-acetylcysteine on sperm capacitation and acrosome reaction in phthalate-treated male Wistar rats

Figure 3A shows the effects of treatment with zinc sulfate and N-acetylcysteine on sperm capacitation in phthalate-treated male Wistar rats. A significant reduction in sperm capacitation was seen in the PHT-treated group when compared to the control ($p < 0.05$).

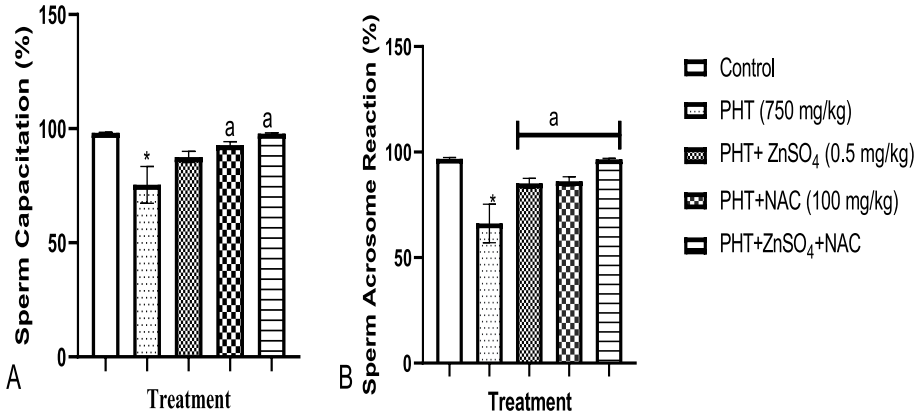

Fig. 3 Effect of treatment with zinc sulfate and N-acetylcysteine on sperm capacitation and acrosome reaction in phthalate-treated male Wistar rats. Values are expressed as mean ± SEM ($n = 5$) (one-way ANOVA followed by Tukey's post hoc test). PHT phthalate, $ZnSO_4$ zinc sulfate, NAC N-acetylcysteine. * and a were considered statistically significant ($p < 0.05$) when compared with the control and PHT-treated groups respectively

There was a significant increase in sperm capacitation [F (4, 20) = 6.070, $p = 0.0023$] in PHT+NAC- and PHT+$ZnSO_4$+NAC-treated groups when compared with the PHT group respectively ($p < 0.05$). Similarly, Fig. 3B shows the effects of treatment with zinc sulfate and N-acetylcysteine on sperm acrosome reaction in phthalate-treated male Wistar rats. As shown in the figure, there was a significant decrease in the percentage of acrosome-intact reacted sperm [F (4, 20) = 8.228, $p = 0.0004$] in the PHT-treated group after incubation in sperm capacitation medium when compared to control groups ($p < 0.05$). On the other hand, there was also a significantly ($p < 0.05$) higher percentage of acrosome-intact reacted sperm in the groups co-treated with PHT+$ZnSO_4$, PHT+NAC, and PHT+$ZnSO_4$+NAC when compared with the PHT-treated group respectively.

Effect of treatment with zinc sulfate and N-acetylcysteine on abnormal sperm chromatin integrity in phthalate-treated male Wistar rats

Figure 4 shows the effects of treatment with $ZnSO_4$ and N-acetylcysteine on sperm chromatin integrity after using toluidine blue stain in phthalate-induced reproductive toxicity in male Wistar rats. Accordingly, treatment with PHT expresses a significant ($p < 0.05$) increase percentage in sperm with abnormal chromatin when compared to control groups. There was also a significant ($p < 0.05$) decrease in the percentage of sperm cells with abnormal chromatin [F

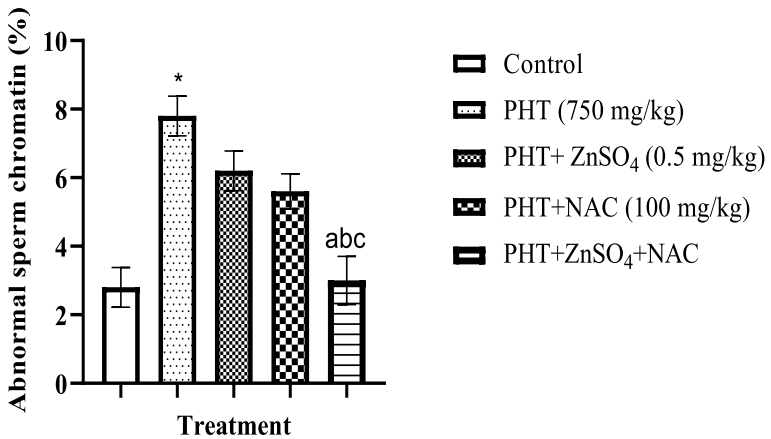

Fig. 4 Effect of treatment with zinc sulfate and N-acetylcysteine on abnormal sperm chromatin condensation in phthalate-treated male Wistar rats. Values are expressed as mean ± SEM ($n = 5$) ($p < 0.05$) (one-way ANOVA followed by Tukey's post hoc test). $n = 5$, PHT phthalate, $ZnSO_4$ zinc sulfate, NAC N-acetylcysteine. *, **a**, **b**, and **c** were considered statistically significant when compared with the control, PHT-, PHT+$ZnSO_4$-, and PHT+NAC-treated groups respectively

(4, 20) = 7.873, $p = 0.0006$] in PHT+$ZnSO_4$+NAC-treated groups when compared with the PHT-, PHT+$ZnSO_4$- and PHT+NAC-treated groups respectively.

Effect of zinc sulfate and N-acetylcysteine on serum electrolyte (Zn and Mg) in phthalate-treated male Wistar rats

The effect of treatment with zinc sulfate and N-acetylcysteine on serum electrolyte (Zn and Mg) in phthalate in phthalate-treated male Wistar rats is shown in Fig. 5. The chronic treatment with phthalate (750 mg/kg) produced a significant ($p<0.05$) decrease in the serum level of Zn (Fig. 5A) and Mg (Fig. 5B) in the groups treated with only PHT when compared with their respective control ($p < 0.05$). However, Fig. 5A also shows a significant ($p<0.05$) increase in the serum level of zinc in the PHT+$ZnSO_4$ and PHT+$ZnSO_4$+NAC when compared with the group treated with only PHT, and Fig. 5B shows a significantly ($p<0.05$) high serum Mg level in the PHT+$ZnSO_4$+NAC-treated group when compared to the PHT treatment group. There was no inter-group difference in the level of Zn and Mg between PHT+$ZnSO_4$+NAC-, PHT+$ZnSO_4$-, and PHT+NAC-treated groups respectively.

Effect of treatment with zinc sulfate and N-acetylcysteine on testicular inflammatory biomarker (TNF-α) and anti-apoptotic factor (BCL-2) in phthalate-treated male Wistar rats

The effect of zinc sulfate and N-acetylcysteine on testicular tissue necrotic factor-alpha (TNF-α) and beta cell lymphoma-2 (BCL-2) in phthalate-treated male Wistar rats is shown in Fig. 6a and b respectively. As demonstrated in Fig. 6a and b, chronic treatment with phthalate (750 mg/kg) produces a significant ($p<0.05$) increase in TNF-α (Fig. 6a) and a significant decrease in BCL-2 (Fig. 6b) in the group treated with only PHT as compared with their corresponding control groups respectively. However, the result also shows a significant ($p<0.05$) lower levels of testicular TNF-α (Fig. 6a) in the PHT+$ZnSO_4$, PHT+NAC, and PHT+$ZnSO_4$+NAC when compared with the group treated with only PHT, with a better outcome in the PHT+$ZnSO_4$+NAC-treated group. In Fig. 6b, there was also a corresponding increase seen in BCL-2 levels in the PHT+$ZnSO_4$+NAC-treated group when compared with the PHT-, PHT+$ZnSO_4$-, and PHT+NAC-treated groups respectively.

Effect of treatment with zinc sulfate and N-acetylcysteine on testicular Ca^{2+} ATPase level in phthalate-treated male Wistar rats

Figure 7 shows the effect of treatment with zinc sulfate and N-acetylcysteine on testicular *Ca^{2+}-ATPaese* level in phthalate-treated male Wistar rats. Accordingly, testicular Ca^{2+}ATPase [F (4, 20) = 15.58, $p < 0.0001$] was significantly reduced in the group treated with only PHT when compared with the control ($p < 0.05$). Testicular *Ca^{2+}-ATPaese* was also significantly increased in PHT+$ZnSO_4$, PHT+NAC, and PHT+$ZnSO_4$+NAC when compared with the PHT-only-treated group respectively. The result also showed that testicular *Ca^{2+}-ATPaese* activities were significantly ($p < 0.05$) higher in the PHT+$ZnSO_4$+NAC group when compared with the other treatment groups respectively.

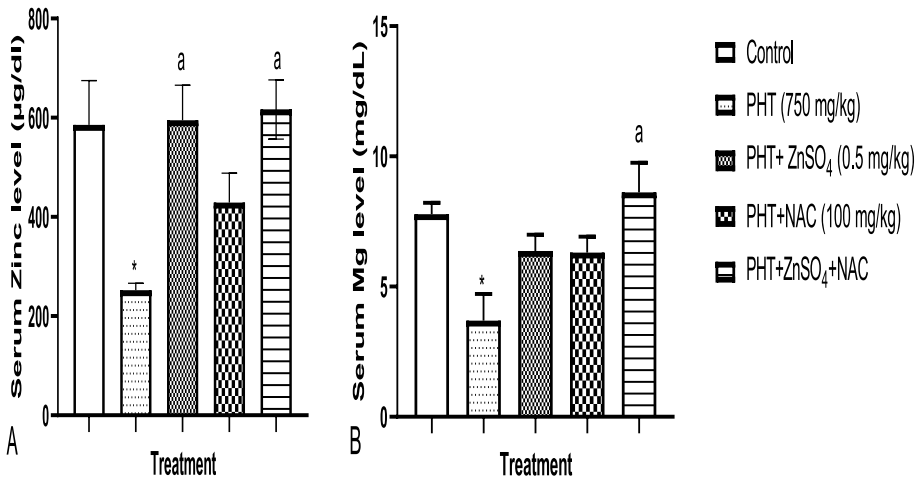

Fig. 5 Effect of co-administration of zinc sulfate and N-acetylcysteine on serum zinc (**A**) and Mg (**B**) levels in phthalate-treated male Wistar rats. Values are expressed as mean ± SEM ($n = 5$) (one-way ANOVA followed by Tukey's post hoc test). PHT phthalate, $ZnSO_4$ zinc sulfate, NAC N-acetylcysteine. * and a $p < 0.05$ considered statistically significant when compared with the control and PHT-treated groups respectively

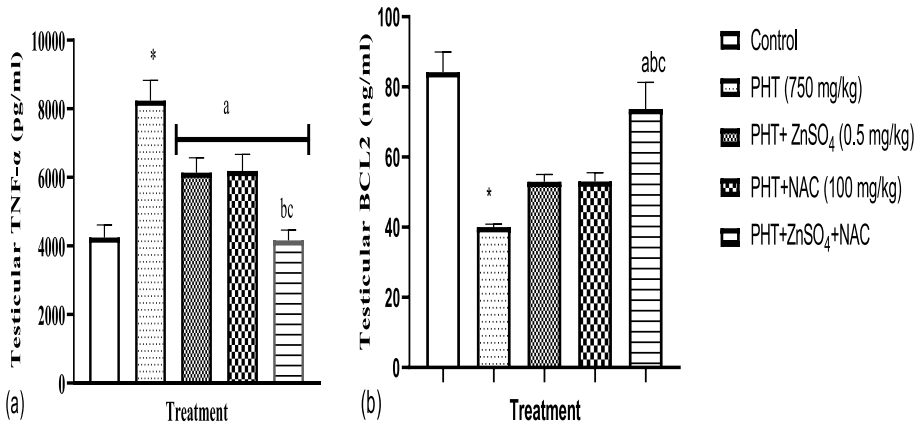

Fig. 6 Effect of zinc sulfate and N-acetylcysteine on testicular inflammatory biomarker (TNF-α) and anti-apoptotic factor (BCL-2) in phthalate-induced reproductive toxicity in male Wistar rats. Values are expressed as mean ± SEM ($n = 5$) (one-way ANOVA followed by Tukey's post hoc test). PHT phthalate, $ZnSO_4$ zinc sulfate, NAC N-acetylcysteine. *, **a**, **b**, and **c** $p < 0.05$ statistically significant when compared with the control, PHT-, PHT+$ZnSO_4$-, and PHT+NAC-treated groups respectively

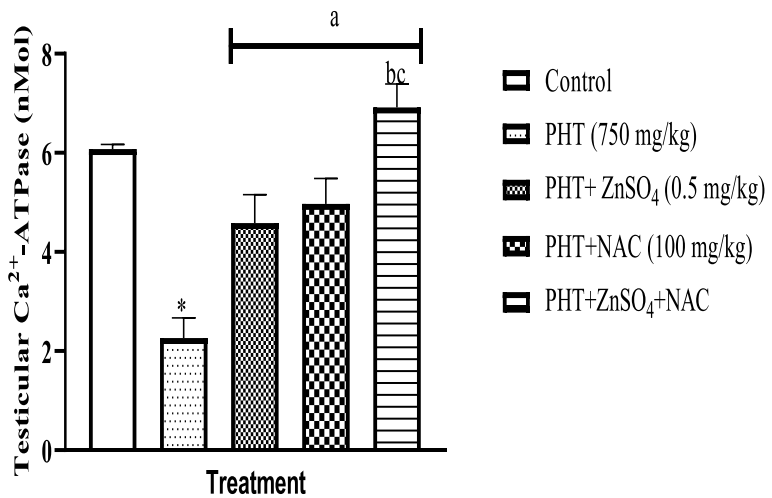

Fig. 7 Effect of treatment with zinc sulfate and N-acetylcysteine on testicular Ca^{2+}ATPase level in phthalate-treated male Wistar rats. Values are expressed as mean ± SEM ($n = 5$) ($p < 0.05$) (one-way ANOVA followed by Tukey's post hoc test). PHT phthalate, $ZnSO_4$ zinc sulfate, NAC N-acetylcysteine. *, **a**, **b**, and **c** were considered statistically significant ($p < 0.05$) when compared with the control, PHT-, PHT+$ZnSO_4$-, and PHT+NAC-treated groups respectively

Effect of treatment with zinc sulfate and N-acetylcysteine on histology of the testis in phthalate-treated male Wistar rats

Plate 1 A shows a testicular tissue from the control group showing the normal testicular architecture. Plate 1B shows a photomicrograph of a testicular section from the PHT-treated group showing a very poor testicular architecture with several severely fibrotic, atrophic seminiferous tubules which exhibit thickened propria enveloping the tubules and some vacuolations. There are also degenerated epithelial germ cells and some seminiferous tubules seen with degeneration and maturation arrest. The interstitial spaces appear normal but also seen as moderately congested tunica albugenia (Plate 1B). A photomicrograph of a testicular section from the group treated with PHT+$ZnSO_4$

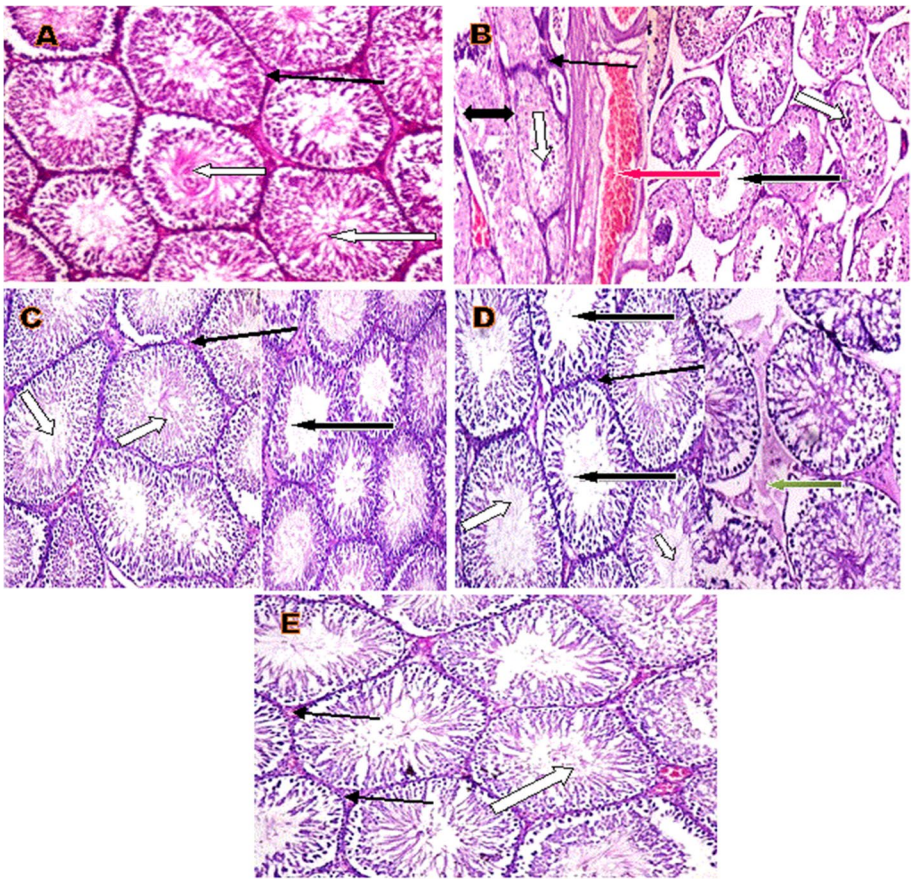

Plate 1 **A** Effect of treatment with zinc sulfate and N-acetylcysteine on histology of the testis in phthalate-treated male Wistar rats. **A** Photomicrograph of testicular sections from the control group: seminiferous tubules with the presence of spermatozoa (white arrow). The interstitial spaces and Leydig cells (slender arrow). **B** Photomicrograph of a testicular section from the PHT group; very poor testicular architecture (spaned black), fibrotic and atrophic seminiferous tubules with and vacuolation (white arrow), seminiferous tubules with degeneration and maturation arrest (black arrow), interstitial spaces (slender arrow), moderately congested tunica albugenia (red cells). **C** Photomicrograph of a testicular section from the PHT+$ZnSO_4$-treated group; seminiferous tubules with the presence of spermatozoa (white arrow). Seminiferous tubules show maturation arrest (black arrow), interstitial spaces with Leydig cells (slender arrow). **D** Photomicrograph of a testicular tissue treated with PHT+NAC; seminiferous tubules with the presence of spermatozoa (white arrow). Seminiferous tubules with maturation arrest (black arrow). Interstitial spaces (slender arrow), congestion (green arrow). **E** Photomicrograph of a testicular section from the group treated with a combination of PHT+$ZnSO_4$+NAC; seminiferous tubules with the presence of spermatozoa (white arrow). Interstitial spaces with normal Leydig cells (slender arrow)

(Plate 1C) shows several normal seminiferous tubules with normal germ cell layer with maturation stages, the lumen appears normal with the presence of spermatozoa but few seminiferous tubules still show maturation arrest while the interstitial spaces and Leydig cells now appear normal. Plate 1D is the photomicrograph of a testicular section from the group co-treated with PHT+NAC showing several normal seminiferous tubules with normal germ cell layer with normal maturation stages, the lumen appears normal with the presence of spermatozoa but there are still few seminiferous tubules with maturation arrest. The interstitial spaces appear normal but still with areas appearing congested. Photomicrograph of a testicular section from the group treated with PHT+$ZnSO_4$+NAC is shown in Plate 1E: the histology shows a normal testicular architecture with normal seminiferous tubules and normal maturation stages with the presence of spermatozoa within their lumen. The interstitial spaces also show normal Leydig cells

Fecundity (%pregnancy, litter size, average pub weight, gestation length) outcome in phthalate-exposed male Wistar rats treated with zinc sulfate and N-acetylcysteine and percentage survival of the offspring of phthalate-treated male Wistar rats after 1 month

Table 1 shows the effects of treatment with zinc sulfate and N-acetylcysteine on fecundity in phthalate-induced reproductive toxicity in male Wistar rats. Table 1 shows a lower percentage (33.3%) of pregnancy outcome in female Wistar rats mated with male Wistar rats treated with phthalate (PHT) only. There was no difference in the pregnancy outcome between female mated with male in the control group (100%) and the PHT+NAC (100)- and PHT+$ZnSO_4$+NAC (100)-treated groups but these values were significant when compared with the PHT group respectively. The result also shows a reduction in litter size in groups mated with male Wistar rats from the PHT group as compared to the control group. However, the litter size in females mated with males treated with PHT+$ZnSO_4$+NAC seems to be higher than that with PHT-, PHT+$ZnSO_4$- and PHT+NAC-treated groups.

Discussion

For toxicological studies, organ weight is the most important criterion [42]. An earlier study of consequences of toxic substances on organs weight has demonstrated that the testis is more sensitive to endocrine disruptors than other important organs in the body [43]. The reduction in weight observed in this study following administration of phthalate is due to degeneration of some vital structures of the epididymis and testis (seminiferous tubules and Leydig cells) as shown in Plate 1. The degeneration of the seminiferous tubule implies a decrease in numbers of germ cells, Sertoli cells, and consequence low semen output that is discussed later in this section. This finding is in line with a similar reduction in organ weight observed in pubertal rats in a previous [44, 45]. However, co-administration of phthalate with either of $ZnSO_4$, NAC, and $ZnSO_4$+NAC was able to ameliorate the effect of phthalate on testicular and epididymis weights. The treatment also ameliorated phthalate-induced epididymis/testicular ratio derangement following its co-administration with $ZnSO_4$ and $ZnSO_4$+NAC. This shows the attenuating potentials of the combination of $ZnSO_4$ and NAC on the testicular and epididymal weight loss possibly due to their cytoprotective potentials.

The quality and fertility potentials of sperm have declined dramatically over the last decade [46–48]. The current study found that phthalate (750 mg/kg) administered alone for 3 weeks reduced sperm count, viability, motility, and increased cells with abnormal morphology. However, when zinc sulfate and N-acetylcysteine or a combination of both was co-administered with phthalate for 21 days, the negative effects of phthalate on sperm quantity and quality were ameliorated. Although this study did not examine ROS, several studies have linked the effects of phthalate on sperm parameters to the generation of reactive oxygen species (ROS) at the cellular level [49–55]. The pathway for causing the damaging effects on spermatogenesis could also be by reducing levels of testosterone due to degeneration of Leydig cells observed in the present study, or early detachment of the germ cells from the Sertoli cells as presented in the histology (Plate 1). Consequently, spermatogenesis (Murphy and Richburg, 2015) was hampered [56]. Although this finding is consistent with our earlier report [6], it is contrary to the report of Tian et al. [57], who noticed a positive association between low-level environmental phthalate exposure and sperm motility. The differences observed here might be due to the adopted doses and duration of exposure. Co-administration of phthalate with $ZnSO_4$, NAC, and $ZnSO_4$+NAC also ameliorated the effects of phthalate on sperm quality and quantity in this study. Although there is no existing evidence on the combined effects of $ZnSO_4$ and NAC on semen parameters, this finding correlates with the report of [58] who reported improvement from different treatments with $ZnSO_4$, NAC, and other antioxidants on sperm indices. Da-Silva et al. [50] earlier reported that the effect of arsenic trioxide on the male mouse genital system was

Table 1 Effects of treatment with zinc sulfate and N-acetylcysteine on fecundity in phthalate-treated male Wistar rats

Group ($n = 6$)	%Pregnancy	Gestation length (days)	Average litter size	Average pup weight (g)	Pop survival rate after 1 month (%)
Control	100	22±0.58	7.0±0.58	10.2±0.35	79
PHT	33.3*	20±0.00	4.0±0.00*	9.9±0.00	100
PHT+ $ZnSO_4$	66.7	24±1.00	6.5±0.50	10.1±0.30	77
PHT+NAC	100[a]	23±1.53	6.0±0.33	9.87±0.17	79
PHT+ $ZnSO_4$+NAC	100[a]	24.5±1.00	8.0±0.58[a]	9.63±0.09	77

PHT phthalate, *$ZnSO_4$* zinc sulfate, *NAC* N-acetylcysteine

*,[a]$p < 0.05$ was considered statistically significant when compared with the control and PHT-treated groups respectively

improved by the co-administration of N-acetylcysteine. Similarly, [59] also reported similar ameliorative activities on co-administration of zinc sulfate and vitamin E on reproductive toxicity caused by aluminum sulfate in male albino rats. We therefore believe that $ZnSO_4$ and NAC could have done these through their anti-oxidative and cytoprotective potentials.

To achieve effective fertilization under normal situations in the oviduct, the mammalian sperm cells must first undergo capacitation [60, 61] followed by acrosome reaction [61, 62]. Therefore, the extent of severity of male infertility depends on the degree of inhibition of acrosomal reaction and sperm capacitation [63]. Using the Coomassie brilliant blue staining technique, phthalate was found to significantly reduce sperm capacitation and acrosome reaction, an effect that was ameliorated by co-administration of $ZnSO_4$ and NAC in the present study. This suggests an ability of the therapy to improve egg binding and fertilization which can be attributed to the cytoprotective effects of this combination and its possible effect on the electrogenic pump as implicated by the higher Ca^{2+}ATPase activities also shown in the PHT+$ZnSO_4$+NAC group in this study. These findings support the existing hypothesis that some forms of phthalate may affect sperm motility, penetration ability, and capacitation [64], and contrary to the report of Sun et al., [65] who observed that neither DEHP nor MEHP alone or in combination had any effect on capacitation following incubation of the sperm sample in a small concentration of phthalates. The difference observed here is attributed to the difference in methods of evaluation and duration of exposure adopted in the studies.

Sperm DNA fragmentation index is an important marker of male infertility while an excessive sperm DNA fragmentation has been linked to poor sperm quality, fertilization process, embryo quality, and pregnancy outcome in previous studies [66, 67]. Consequently, the result of the present study showed an increase in abnormal sperm chromatin in the group treated with only phthalate for 21 days. This is an indication that phthalate may directly attack sperm DNA by altering chromatin level, thereby leading to a high level of abnormal spermatozoa also noticed in this study. The use of antioxidants has been linked with the amelioration of negative impacts of chemo toxicants on sperm DNA [68, 69]. Similarly, NAC and $ZnSO_4$ co-administered with phthalate ameliorated the negative impact of phthalate in the present study. This is due to the antioxidant ability of NAC and $ZnSO_4$ and their abilities to maintain cellular levels of Zn and Mg, which are important regulators of DNA replication, transcription, and protein synthesis, influencing cell division and differentiation as earlier stated in previous studies [70, 71]. The outcome of this study is similar to that of Sooklert et al. [72] in which NAC reversed the decrease of DNA methylation status caused by engineered gold, silicon, and chitosan nanoparticles and that of Düzenli et al. [73] in which acetyl-L-carnitine (ALC) and NAC combination treatment inhibits DNA damage and induces DNA repair. Again Baetas et al. [74] who worked on the protective role of N-acetylcysteine on human sperm exposed to etoposide also observed that NAC counteracted the cytotoxic effects of etoposide on sperm DNA, while Jannatifar et al. [75] and Yildiz et al. [76] in their separate studies observed that DNA fragmentation significantly decreases in spermatozoa after NAC treatment. In the same way, an earlier study on zinc showed that a moderate increase in dietary zinc reduces DNA strand breaks in leukocytes [77].

Zinc has been noted to be the second most abundant trace element in humans with many unique properties in the male reproductive system. It is an anti-inflammatory factor and involved in the sperm's oxidative metabolism, a hormone balancer which helps to regulate hormones such as testosterone; it is essential for maintaining the lining of the reproductive organs and also has a regulative role in the progress of capacitation, acrosome reaction, and sperm DNA integrity [78, 79]. Its deficiency prevents spermatogenesis which is a source of sperm defects and has a detrimental effect on the concentration of serum testosterone [70]. Therefore, the fall in serum level of Zn observed in this study after treatment with phthalate is a confirmation that electrolyte imbalance is one of the mechanisms by which phthalate reduced motility and sperm count and increased TNF-α and DNA damage seen in this study. Phthalate used in this study also caused low serum magnesium levels in consistence with the findings of Deger and Akkus [80] who reported lower seminal fluid magnesium levels in different forms of infertile subjects. The effect of phthalate on zinc and magnesium was ameliorated by $ZnSO_4$, NAC, and a combination of $ZnSO_4$+NAC when co-administered with phthalate. The mechanism of action of these substances is through their abilities to improve proton-pump activities.

One mechanism of action of phthalates also observed in this study is inflammation and apoptosis through the mitochondrial apoptotic activities. Two signaling pathways (extrinsic and intrinsic) have been identified to instigate cellular apoptosis. The extrinsic pathway is believed to occur through the action of inflammatory markers such as tumor necrosis factor (TNF) superfamily of ligands binding to their associated receptors while the intrinsic signaling is through events that result in the release of cytochrome C from the mitochondria normally indicated by the low level of BCL-2 [81–85]. The present study showed that exposure to phthalate leads to an increase in the production of testicular inflammatory biomarker-tumor necrotic

factor-alpha (TNF-α) and a fall in testicular level BCL-2. This is an indication that phthalate also exerted its reprotoxic effects via the extrinsic and intrinsic signaling mitochondria pathway normally mediated by oxidative stress. When either of these happens, the Leydig cells, Sertolic cells, and germ cells undergo apoptosis [86–88] which in turn lead to degeneration and sloughing of the cell and thereby leading to poor testicular functions also noted following treatment with phthalate in this study.

However, co-treatment with $ZnSO_4$, NAC, and $ZnSO_4$+NAC was able to ameliorate these effects of phthalate on testicular mitochondria activities by acting as anti-apoptotic agents [78, 88–92].

The result from this study also implicated phthalate in reducing litter size and percentage pregnancy. The effects on litter size and pregnancy outcome are associated with testicular atrophy, reduced epididymal sperm density and motility, and increased numbers of abnormal sperm in male rats as earlier reported in the study of Rowdhwal and Chen [93] and David [94]. Concerning the potential role of co-administration of $ZnSO_4$ and NAC on fertility outcome in phthalate-exposed animals, the fecundity test performed in this study revealed that both zinc sulfate and N-acetylcysteine, administered separately or together, were able to reduce the effects of phthalate on percentage pregnancy and litter size, with a better outcome observed when they were administered together.

Conclusions

Conclusively, the study provides an insight into the mode of action of phthalate on testicular damage and the beneficial role provided by the combined treatment of NAC and $ZnSO_4$. Cumulatively, zinc sulfate and N-acetylcysteine ameliorated the effects of phthalate on testicular functions and increased fertility outcome in male Wistar rats via a mechanism related to the enhancement of testicular BCL-2, inhibition of upregulation of TNF-α, electrolyte balance, stabilization of testicular Ca^{2+}ATPase pumps, cytoprotection, and restoration of spermatogenesis.

Abbreviations

BCL-2: Beta cell lymphoma 2; NAC: N-acetylcysteine; TNF-α: Tissue necrotic factor-alpha; $ZnSO_4$: Zinc sulfate; Ca^{2+}: Calcium; PHT: Phthalate.

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Authors' contributions

AON and EKN conceived and designed and supervised the research; VE conducted experiments, collected samples, and contributed reagents and analytical tools; and MOO analyzed the data while BB edited the article. All authors supplied laboratory resources and edited and approved the final manuscript.

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Availability of data and materials

All data used for this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate

The experimental procedures were approved by the Delta State University Animal Care and Use Research Ethics Committee (REC/FBMS/DELSU/18/05), and the study was performed in accordance with the care and use of Laboratory Animals of the NIH Guidelines by careful handling.

Consent for publication

Not required

Competing interests

The authors declare that they have no competing interests.

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