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DEHP Induced Oxidative Stress Suppress Sperm Quality and Serum Androgen Concentration in Wistar Rats

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Abstract:- World health organization estimates a global infertility prevalent rate of 10-15%. Male factor constitute about 40-50% of infertility cases. Exposure to environmental pollutants such as di-(2-ethylhexyl) phthalate (DEHP) adversely affects reproductive system tissue differentiation and functions, thereby potentiating male infertility. This study was designed to investigate the impact of oxidative stress induced by DEHP on some semen parameters, as well as serum androgen concentration in adult Wistar rats. 40 adult male Wistar rats weighing between 156-250g were randomised into 4 experimental groups 1, 2, 3 and 4 (group *n*=10). Animals in groups 1, 2 and 3 were treated with 0.02mg, 20mg, 200mg oral DEHP/kg bw daily respectively, while those in group 4 served as Control and were treated with the vehicle. All treatments lasted for 60 days. After an overnight fast, samples of semen and serum were obtained for analysis. Results obtained, expressed as mean ± standard deviation, showed that treatment groups exposed to varying doses of oral DEHP had significant ($p<0.05$) reduction in sperm count, total sperm motility, active sperm motility, normal sperm morphology, serum testosterone concentration and serum super oxide dismutase units at values of $31.70\pm18.68x10^6$ cells/mL, 38.60±24.78%, 8.50±5.66%, 38.00±18.00%, 9.56±1.34ng/mL and 0.017±0.0013 units respectively. Sluggish sperm motility and abnormal sperm morphology significantly increased to 39.70±13.05% and 68.50±18.42% respectively. Arising from the findings of this study, it is hereby concluded that oral DEHP potentially suppresses spermatogenesis in adult life by induction of oxidative stress and depressing androgen synthesis, thereby impacting negatively on male fertility.

Keywords:- *Infertility, DI-(2-Ethylhexyl) Phthalate, Oxidative Stress, Semen Quality.*

I. INTRODUCTION

There has been growing concern about declining fertility worldwide. The world health organisation (WHO) fact sheet of 2020 suggest that between 48 million couples and 186 million individuals live with infertility globally; at 15% prevalence rate while in developing countries, the infertility prevalence stands at 25%. Of this figure, 30% is attributable female and male factors alike; 30% and 10% attributed to combine sex factor and unknown factors respectively (1). In Nigeria, male infertility prevalent rate ranges from 25-40% (2), but this figure varies from one part of the country to the other (3). The growing incidence of infertility has generated a great concern in the medical world. In recent times there has been a decline in the semen quality of young healthy men worldwide of non-infective aetiology, with similar findings being reported in Nigeria. This semen quality (indicated by sperm count, motility as well as morphology) decline has significant associations with exposures to heavy metals such as cadmium and lead, mycotoxins such as aflatoxins, pesticides, industrial chemicals and endocrine factors (4). A number of environmental pollutants/toxicants may be classified as environmental factors in the aetiology of infertility. Exposures to environmental toxicants arising from some of these industrial chemicals not only alter spermatogenesis or sperm functions, but are known to inflict diverse reproductive injuries (5).

The relationship between male infertility and Di-(2-ethylhexyl) phthalate (DEHP) have been well established, as the latter has been known to be implicated in diverse forms of male gonadotoxicity (6,7). This compound has been identified in the blood, urine and semen of exposed men, affecting semen/spermatozoa quality (8). DEHP, (also known as bis-(2-ethylhexyl) phthalate (BEHP) or di-octyl phthalate (DOP)), is an organic, colorless and almost odourless liquid, expressed chemically as C_6H_4 $(C_8H_{17}C00)_2$, with a molecular weight of 390.57 g/mol. It is synthesized by the reaction of phthalic anhydride with 2-ethylhexanol and metabolized by hydrolysis to mono-(2-ethylhexyl) phthalate (MEHP)/phthalate salt, leading to release of alcohol which can be oxidized to an aldelyde (9). The breakdown products of DEHP may be measured in urine or blood, an indicator of recent exposure to the compound.

DEHP is the most common plasticizer in polyvinyl chloride (PVC) or plastic, easily leaching out of PVCs. It is a constituent compound in hydraulic fluids, dielectric fluid in capacitors and as solvent in lipsticks. Given the wide use of PVC, ranging from domestic, industrial to medical uses, there exist a widespread exposure to this compound. Due to its highly hydrophobic character, it leaches (extract) rapidly into non-polar solvents compared to polar solvents like water. This property is both time and use dependent. Evidence suggests the general population is exposed to about 2 mg/day of DEHP (10). The most probable route of this exposure is

from plastics used in processing and storing foods (about 0.25mg per day), while the amount found in drinking water ranges from 0.04 to 30 parts per billion (ppb). Inaddition, exposure to DEHP occur during certain medical procedures such as blood transfusions, kidney dialysis and use of respirators (because of their PVC components), with resultant tissue deposition (11,12).

DEHP exerts toxic effects both on the body and the environment in a number of invivo and invitro animal studies (13,14,15). MEHP, one of the metabolites of the DEHP, identified in exposed organisms and has been implicated in the possibility of obesity epidemic (16). At Low dose, DEHP caused significant inhibition of membrane Na^+-K^+ ATPase in brain, liver and red blood cells (RBCs (17) on the one hand and decrease of serum insulin, increase of blood glucose, decrease in liver glycogen, increase in T_3 and T_4 and decrease in cortisol (18) in rats.

Reproductively, exposure to oral DEHP has been demonstrated to cause developmental toxicity (19) such as birth defects in rats and mice; an action of DEHP which is dose, time and exposure age dependent. The risk is relatively higher for developing fetus and newborns, (particularly in the preterm) (20). Other reproductive derangements observed in animals with oral exposure to DEHP include decrease in fertility and proportion of pups born alive, testicular weight reduction and tubular atrophy (10), thus adversely affecting reproductive organ developments in the early stages of life and consequently impacting negatively on male fertility. Owing to these multiple negative actions of DEHP, this study was designed to investigate possible effect(s) of DEHP on the reproductive system of adult Wistar rats.

II. MATERIALS AND METHODS

➢ *Animal procurement and treatment*

Fourty (40) healthy reproductively matured male albino Wister rats weighing between 156-250g (mean weight of 175g) were procured from the Animal house, the College of Health Sciences, Benue State University for the study. They were randomised into 4 experimental blocks 1, 2, 3 and 4 (group *n=10*), and housed in locally constructed wooden cages with wood chip beddings. The rats were acclimatized for two weeks in a maintained controlled environment with a 12hr light/dark cycle. The animals were exposed to free growers chow and water *ad libitum.* Care of the rats was consistent with international animal care regulations (21).

DEHP (Sigma-Aldrich®) was procured from a reputable company outlet and administered as follows:
Group 1- 0.02mg DEHP/kg bw
Group 2- 20mg DEHP/kg bw
Group 3- 200mg DEHP/kg bw.
Group 4- Control

All treatments were administered intrapharyngeally daily for 30 days. On day 31 and after an overnight fast, the animals were observed for general physical activity, mobility/agility and anesthetized with inhalational chloroform. Cardiac puncture was performed (22) to aspirate 3ml of blood into a plain, clean blood sample bottle for assay of serum testosterone concentration and SOD level. One of the testes was randomly selected removed through a clean scrotal incision and careful dissection. This testis was preserved in a test tube containing 0.1ml 0.95% saline solution for semen analysis.

Analysis of Semen was performed on semen obtained from the caudal region of the epididymis by gentle milking in prepared 0.1mL 0.95% saline solution for sperm count, motility and morphology using standardized method in line with WHO protocol for semen analysis (23,24).

Testosterone assay was performed the blood sample collected after been allowed to stand for 40 minutes and centrifuged at a speed of . Using a calibrated pipette, between 0.5 and 1mL of the clear supernatant serum was aspirated into another plain clean blood sample bottle with which testosterone assay was conducted using standard enzyme linked immune sorbent assay (ELISA) method (AccuBind™ Elisa microwell testosterone test system kit, product code 3725-300). The result was read using the Stat fax-2100 microplate reader (Awareness Technology™).

Serum Superoxide Dismutase (SOD) Assay was carried out using the technique described by Marklund and Marklund (25) and adapted in a later study (26) which utilised the rapid auto-oxidation of pyrogallol in aqueous or alkaline medium solution (typified by Tris buffer at pH of 8) as the underlinig principle. The extent of oxidation of pyrogallol is read at a particular wavelength using spectrophotometer. SOD, as an antioxidant, inhibits this auto oxidation of pyrogallol. The degree of oxidation of pyrogallol is inversely proportional to the amount of SOD present in the serum.

➢ *Statistical analysis*

Data obtained were entered into Microsoft® office excel spread sheet version 2010 and expressed as mean ± SD. The data was then analyzed using Qi Macros 2020. One way analysis of variance (ANOVA) was employed to test significant differences between and within group means. Significant difference in means was tested using t-test at 95% confidence limit. Where significant differences exist among mean, Post Hoc test was applied to define specific level of group mean differences.

III. RESULTS

Results obtained from the study at the end of treatment indicated that the parameters studied were adversely affected in the adult Wistar rat on exposure to oral DEHP, correlating with SOD suppression in serum.

➢ *Sperm count*

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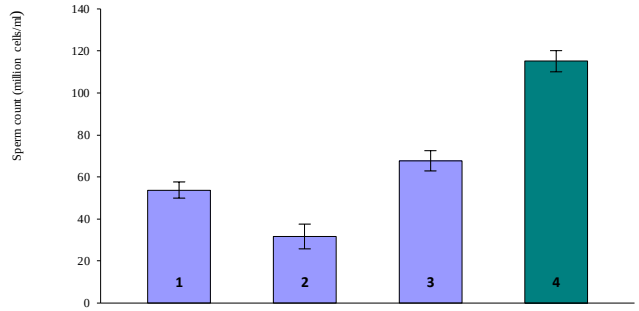

Fig 1: Effect of oral DEHP exposure on Sperm count in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3

Control group

Figure 1 shows the response of sperm count to oral DEHP treatments in the experimental animals. There was significant reduction (*p<0.01)* in mean sperm count in groups 1, 2 and 3 (53.80±12.10, 31.70±18.68 and 67.70±15.30 million cells/mL respectively) relative to the mean sperm count of 115.10±15.95 million cells/mL of group 4. This implies that oral exposure to DEHP induces suppression of total sperm count in the adult Wistar rats.

➢ *Sperm motility*

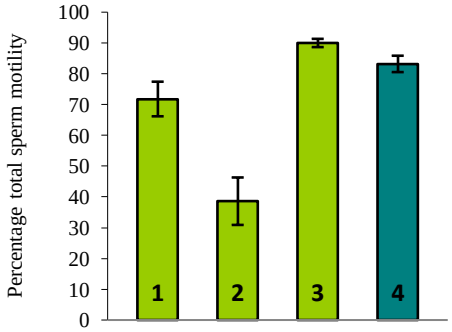

Fig 2: Effect of oral DEHP exposure on Total sperm motility in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3

Control group

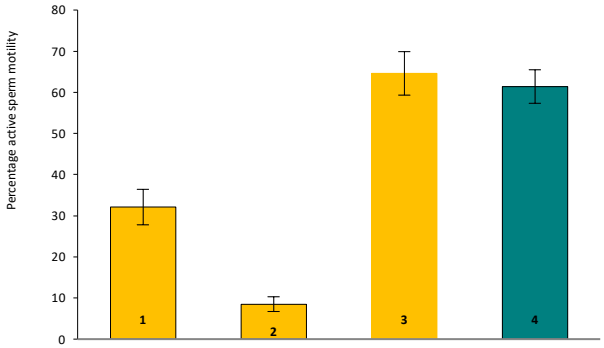

Fig 3: Effect of oral DEHP Active sperm motility in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3

Control

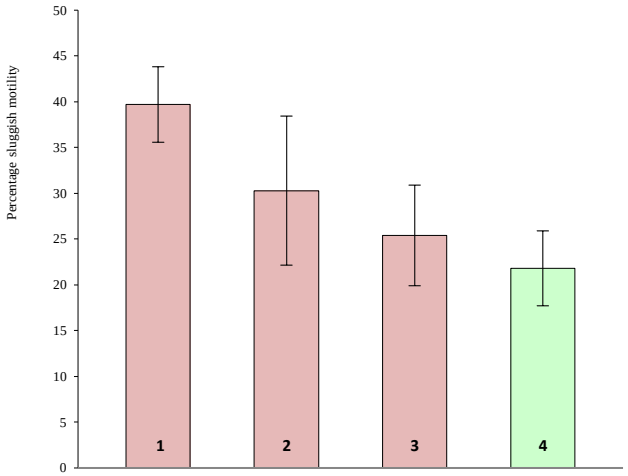

Fig 4: Effect of oral DEHP exposure on Sluggish sperm motility in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3

Control group

The effects of oral treatment on sperm motility are shown in figures 2, 3 and 4. In figure 2, mean total sperm motility was reduced significantly (*p<0.05*) to 38.60±24.78% only in group 2 from 83.20±8.42% in the Control group (a 2.2 fold reduction), while the mean total sperm motility of groups 1 and 3 was not significantly different (*p<0.05*) compared to the Control group respectively.

In figure 3, mean active sperm motility was reduced significantly (*p<0.05)* only in groups 1 and 2 to 32.10±13.66% and 8.50±5.66% respectively from 61.40±12.88% of Control group. This reduction was comparatively more profound in group 2.

In figure 4, mean mean sluggish sperm motility in groups 1, 2 and 3 were 39.70±13.48%, 30.30±25.77% and 25.40±17.39% respectively compared to 21.80±12.94% of the Control. This showed a numerical increase in the population of sluggishly motile/immotile spermatozoa, but significant increase (*p<0.05*) was observed only in group 1 relative to the Control group.

➢ *Sperm morphology*

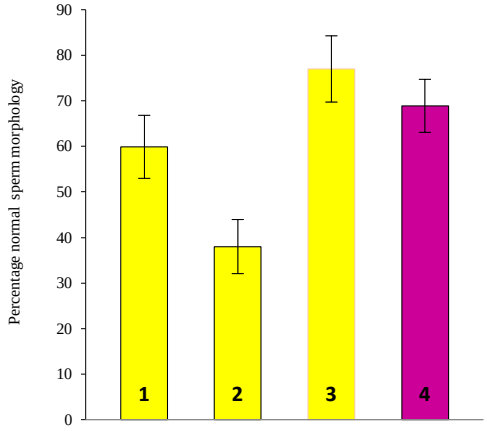

Fig 5: Effect of oral DEHP exposure on normal sperm morphology in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3
Control group

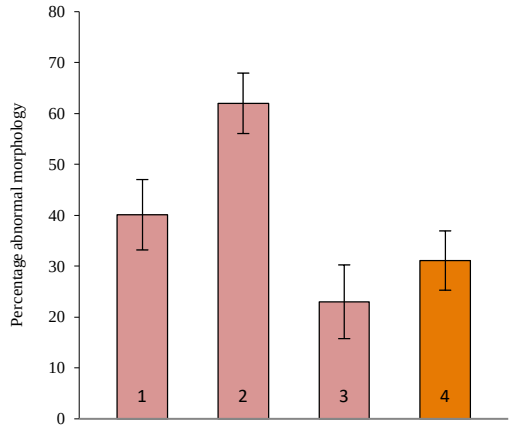

Fig 6: Effect of oral DEHP exposure on abnormal sperm morphology in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3
Control group

In figure 5, the treatment effects of oral DEHP on sperm morphology were shown. Sperm cells with mean normal sperm morphology of 59.90±21.89%, 38.00±18.00 % and 77.00 ± 22.88 % respectively in groups 1, 2 and 3. This indicated a significant (*p<0.01)* reduction (1.8 folds) in normal sperm morphology in group 2 from 68.90±18.42% of the Control group. On the other hand, the mean abnormal sperm morphology were 40.10±21.89%, 62.00±18.80% and 23.00±22.88% in groups 1, 2, and 3 respectively, indicating a significant (*p<0.05)* increase (about 2 folds) only in group 2, compared with 31.10±18.41% of Control group (figure 6). This is an indication that DEHP enhance the synthesis of more abnormal sperm cells due spermatogenesis.

➢ *Serum Testosterone Concentration*

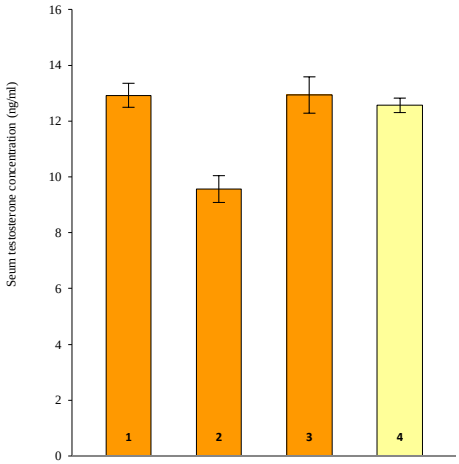

Fig 7: Effect of oral DEHP on Serum testosterone concentration in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3
Control group

In figure 7, mean serum testosterone concentration in groups 1, 2 and 3 treated with oral DEHP only were 12.92±1.52ng/mL, 9.56±1.34ng/mL and 12.15±2.04ng/mL respectively, reflecting a significant reduction (*p<0.05)* only in group 2 compared to 12.56±0.82ng/mL, implying that oral DEHP exposure reduces serum testosterone concentration.

➢ *Serum Super oxide dismutase concentration*

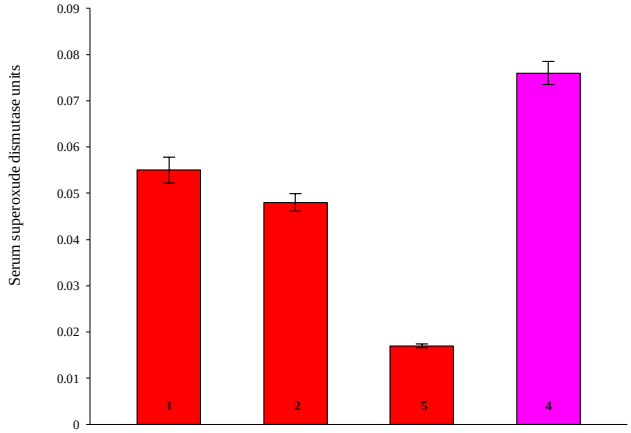

Fig 8: Effect of DEHP serum Super oxide dismutase units in adult Wistar rats

Key

DEHP treatment in groups 1, 2, 3
Control group

At the end of treatments, the mean serum SOD level (units/mL) were 0.055±0.010, 0.048±0.010 and 0.017±0.000 in experimental groups 1, 2 and 3 (figure 8). Comparing with the Control group value of 0.076±0.010 units/mL, significant reductions *(p<0.05*) were observed in groups1, 2 and 3. This implies that exposure to oral DEHP induces oxidative stress in the adult reproductive tissues (indicated by reduction in serum SOD.

IV. DISCUSSION

Exposure to DEHP, classified as endocrine disruptor (EP), directly impairs spermatogenesis through the generation of oxidative stress in the gonads (27). In the present study, it has been shown that exposure of rats to DEHP resulted in significant alteration in semen parameters (sperm count, motility and morphology) in the adult Wistar rats, indicating some degree of gonadotoxicity in the male. This finding is consistent with earlier studies (28,29) which described evidence of cellular disruption in the testes of Wistar rats exposed to DEHP, resulting in derangements of semen profile and testicular histology characterised by clustered multinucleated gonocytes, aggregation/clumping of sertoli cells with evidence of seminiferous tubular necrosis. These indicate that DEHP targets and disrupts the spermatogenic process in the testes. This altered outcome of spermatogenesis may arise from the suppression of serum testosterone concentration, as seen in the current study.

A possible mechanism of action of DEHP in disrupting spermatogenesis is through suppression of testosterone synthesis. There was evidence of significant reduction of serum testosterone concentration in DEHP exposed rats. This is consistent to earlier study (30). The maintenance of successful spermatogenesis is dependent on sufficiently high local testosterone hormone sequestration in the testes, although the precise mechanism for this sequestration remains a subject of studies (31). The maintenance of such high level of testosterone synthesis and sequestration by Leydig cell (LC) rquire abundant supply of cholesterol, which can either be imported from the extracellular space or synthesized intracellularly from lipids. Therefore, lack of adequate supply of cholesterol in the testes may affect the synthesis of testosterone and its subsequent sequestration. Pre-pubertal female rats exposed to inhalational DEHP have shown evidence of decreased serum cholesterol level in adulthood. This suppression of serum cholesterol, and consequently, serum testosterone concentrations may be related to the ability of DEHP to preferentially and selectively target *Scarb1* and *Star* genes involved in transporting cholesterol on the one hand and *Cyp11a1* gene (also known as *P450scc, Hsd3b1,* and *Cyp17a1*) involved in converting cholesterol to testosterone on the other hand (32). These genes are involved in pathways that directly and indirectly affect testosterone production by the LCs as well as those that may be essential in the role testosterone plays in the normal interaction and development between SCs and the maturing spermatocytes. Targeting these genes by DEHP therefore, may impair these pathways and consequently negatively affect spermatogenesis qualitatively and quantitatively. Inhibition of testosterone production in the testes (hence depress serum testosterone concentration) may be occur through the angiotensin and vasopressin inhibitory pathway. DEHP and phthalates in general induces this dual angiotensin and vasopressin receptor (*Nalp6*), (together with aminopeptidase A, an enzyme responsible for converting angiotensin II to angiotensin III) in the testes, causing inhibition of testosterone production by the LCs (33). This depressed serum, and particularly, testicular testosterone concentrations are consequentially deleterious to the process of spermatogenesis which is associated with the detachment of developing spermatids, especially between steps 7-8 of spermatogenesis from the SCs, thereby halting spermatogenesis during the process of meiosis. The inadequacy of testosterone sequestration in the testes is due to failure of SCs to produce the adhesion molecule N-cadherin, the production of which appears to require both FSH and testosterone. Furthermore, the formation of blood testes barrier (BTB) is compromised resulting in the premature displacement of immature germ cells from the SCs on the one hand, and the inability of mature spermatozoa to be released from the SCs on the other hand. Thus, the DEHP induced suppression of serum testosterone concentration in exposed rats may be due to interference in the steroidogenic as well as inhibition of testosterone synthetic pathways respectively.

In the current study, there was significant depression of serum SOD in DEHP exposed rats. This finding of dose dependent depression of serum SOD is consistent with the finding of earlier study (34). Other studies (35,36) have shown that the induction of oxidative stress in tissues is associated with reduction in levels of SOD. Serum SOD level is related to reactive oxygen specie (ROS) index in an inverse proportionality; a reduction in the former is associated with a rise in the latter. Thus, serum SOD levels may be a useful marker of tissue oxidative stress. In the body, though ROS are formed as a natural by product of normal oxidative metabolic process
which play important role in cell signaling and homeostasis, the ROS levels can increase significantly resulting in significant damage to cell structures (the phenomenon of oxidative stress). Most body cells, and spermatozoa particularly, are susceptible to the activities of oxidants (in oxidative stress) since their membranes posses high amounts of polyunsaturated fats which can easily be rendered unusable when oxidized into saturated fats. However, the levels of these radical generated in the body are kept at acceptable (useful) levels by a group of scavengers known as cellular antioxidant enzymes which include SOD, peroxidases (gluthatione proxidase especially), reductases and catalase amongst others. SOD is associated with a host of activities in the human body especially that which is responsible for mopping up of oxidizing radicals like ROS with attendant significant reduction of the serum level of the enzyme (26). The activities of SOD are linked to the activities of peroxisome proliferator-activated receptors (PPARs). There is a positive correlation between PPAR protein expression and SOD activity in tissues (37). The main physiological role of PPARs is to transform various environmental, nutritional or inflammatory stimuli into intracellular signals that regulate lipid metabolism, cell proliferation and differentiation. DEHP alters the transactivation of α subunit of PPAR leading to induction of subsequent transcriptional changes with a broad range of effects on reproductive cell function (38).

DEHP or its metabolite MEHP, induces the proliferation of peroxisome, a process resulting in the possibility of enhanced production of ROS which are chemically reactive chemical species containing oxygen such as the hydrogen peroxides (H_2O_2), superoxides (O_2^-), hydroxyl radicals (OH^-), singlet oxygen radical (O^-) which are capable

of freely and easily donating an oxygen radical, thereby acting as powerful oxidants. In the gonads, DEHP induces oxidative stress by generating high levels of ROS (39,40,41). This action is associated with decreased testosterone levels as well as increased incidence of multinucleated gonocytes (spermatocytes) in seminiferous cords (42) due to the direct effect of ROS which induces defective spermatozoa DNA formation, targeting JAZF1, TR4, Sperm 1, and Cyclin A1 genes. Spermatozoa are highly susceptible to reactive oxygen species (ROS), which are key intermediates in cellular signal transduction pathways (4), and whose actions may be counterbalanced by antioxidants. This action is mediated by *Bmi1*, the polycomb repressor universally expressed in all types of testicular cells. *Bmi1* deficiency resulted in ROS accumulation and oxidative tissue and DNA damage (43).

V. CONCLUSION

The study has shown that oral exposure to DEHP impacts negatively on the spermatogenesis qualitatively and quantatively in the adult rat gonads through the induction of oxidative stress and possibly through interference with testicular steroidogenic pathways resulting in inhibition of testosterone production, thereby enhancing male infertility.

RECOMMENDATION

Arising from the findings of this study, we recommended that attempts should be made in reducing exposure of male animals of all ages to DEHP by de-emphasising dependence on use of plasticized PVC materials, especially for domestic and medical purposes. We equally recommend the supplementation of antioxidants in diets to enable the body combat oxidative stressors more effectively which can promote male fertility.

LIMITATION

Even though the study was well designed to satisfy the overall aim, the study was limited in scope owing to economic and time constraints.

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CONFLICT OF INTERESTS

We the authors hereby declare that this original work has not been previously published, nor is it before another journal for consideration.

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