

Research Article

Glyphosate, Chronic Toxicity on Male Reproductive Indices and The Role of Zinc Supplementation in Wistar Rats

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Abstract

Sixty-four adult male Wistar rats were used. They were randomized into eight (8) groups viz: Group I administered distilled water (2 ml/kg), group II, administered zinc (50 mg/kg), group III administered glyphosate (14.4 mg/kg), group IV, administered glyphosate (375 mg/kg), group V administered glyphosate (750 mg/kg), group VI pretreated with zinc (50 mg/kg) for an hour then glyphosate administered (14.4 mg/kg), group VII, pretreated with zinc (50 mg/kg) for an hour then glyphosate administered (375 mg/kg) and group VIII, pretreated with zinc (100 mg/kg) for an hour then glyphosate administered (750 mg/kg). The experiment lasted for 36 weeks. At the end, blood samples from four rats in each group were collected after chloroform anesthesia and used for serum hormonal assays and samples of testes were taken for seminal and sialic acid analyses. Semen characteristics showed relative decreases in sperm concentration, live sperm concentration, sperm motility, progressive sperm motility and significant decrease in normal sperm morphology. Sperm tail abnormality, dead sperm and non-motile sperm motility relatively increased and significant increases were recorded in abnormal sperm morphology and non-progressive sperm motility and variable changes were obtained in mid-piece abnormality; decreased sialic acid concentration was recorded. Relative decrease in serum testosterone and marginal increases in serum luteinizing and follicle stimulating hormone were recorded. Fat vacuoles in the seminiferous tubules, degeneration of the spermatogenic cells, abnormal spermatids were recorded. It was concluded that the changes sequel to chronic glyphosate exposure in male Westar rats in this study were ameliorated with zinc.

Keywords: Chronic; Glyphosate; Reproductive Indices; Zinc

Introduction

Glyphosate is the active ingredient of all the glyphosate-based herbicides which are broad spectrum herbicides that mediate their phytotoxic action by inhibiting the synthesis of protein through inhibition of shikimic acid pathway [1]. In mammals, the mechanism of toxic action of glyphosate is still not clear and may have several enzymatic effects [2,3], however, some agricultural

workers using glyphosate-based herbicides were reported to have pregnancy problems [4] and reduced respiratory control ratio, enhanced ATPase activity and stimulated oxygen uptake rate were observed in liver- mitochondria of rats given glyphosate and these toxicological effects were said to be primarily as a result of uncoupling of oxidative phosphorylation [5].

Oxidative stress was reported to be involved in toxicity of glyphosate at molecular level [6]. The body reacts to oxidative stress by evoking the enzymatic defense system in the body [7].

There is paucity of information on the reproductive effects of glyphosate and the few available literatures are at variance with one another. Laboratory studies have found that glyphosate and round-up® formulations may be linked to endocrine disruption in animals and human cell lines [8-11] with effects recorded at concentrations below those used in agriculture. Whether or not such effects could be occurring in wildlife after field application of glyphosate has not yet been established and glyphosate is not currently included on lists of confirmed endocrine disrupting chemicals (UHPPDB) [12] whereas SERA [13] documented that, tests on the potential effect of glyphosate on the endocrine system have been conducted and all of these tests reported no effect and they deduced that, conclusion on glyphosate not been an endocrine disruptor is reinforced by epidemiological studies that have examined relationships between occupational exposures to glyphosate formulations and risk of spontaneous miscarriage, fecundity, sperm quality and serum reproductive hormone concentrations and the studies have not found positive associations between exposure to glyphosate formulation and reproductive or endocrine outcomes.

Living organisms contain a complex network of antioxidant metabolites and enzymes that work synergistically to avoid oxidative damage to cellular components such as DNA, proteins and lipids within the body [14,15]. The antioxidant systems generally either prevent reactive oxygen species formation or eliminate them before they become detrimental to important cell components [14,15]. The reactive oxygen species play a vital role in cellular signaling and therefore, their function is not to remove oxidants entirely but rather to keep them at optimum level [16].

Zinc is an essential trace element for a number of animal species [17-19]. Zinc performs its antioxidant role through two essential mechanisms as follows: The first mechanism is the protection of sulfhydryl groups from oxidation and this it does by preventing intramolecular disulfide formation [20]. The second mechanism is by the prevention of free radicals (-OH and -O₂) formation by transition metals [21]. Since oxidative stress was reported to be involved in glyphosate toxicity [6], it was inferred that an antioxidant will play an important role in mitigating the pathological changes if any following chronic exposure to graded doses of glyphosate in this study.

Materials and Methods

Research Animals: Acquisition, Acclimation, Housing and Feeding

Eighty adult male Westar rats used for this experiment were purchased from the National Institute for Trypanosomiasis and Onchocerciasis Research, Vom Office, Jos Plateau State. They were kept in the animal room of the Department of Veterinary Pathology, Ahmadu Bello University-Zaria, Nigeria for two weeks for acclimatization before the commencement of the research. The rats were dewormed during the two weeks using albendazole at

the dose rate of 10 mg/kg body weight as reported by Teruel [22]. The rats were fed using standard rat chow and water was given ad libitum.

Compliance with Ethical Standards

This work was carried out after approval was obtained from the ethical committee on the care and use of animals in research of Ahmadu Bello University, Zaria, Nigeria.

Chemicals and Test Kits Sources

Glyphosate (Bushfire®) which contains 360g glyphosate/liter in the form of 441g/liter potassium salt, distilled water, hematoxylin and eosin stain, Sudan black stain, chloroform and zinc chloride (BDH Chemical Ltd; Poole, England) is a white deliquescent granule with minimum assay 98.0 %, maximum limits of impurities: Acid-insoluble matter 0.005 %, zinc oxide 1.2 %, sulphate 0.002 %, cadmium 0.0005 %, calcium 0.001 %, copper 0.0005 %, iron 0.001 %, lead 0.001 %, magnesium 0.001 %, potassium 0.001 % and sodium 0.001 % were purchased from a reputable chemical store in Zaria. Enzyme immunoassay kits for follicle stimulating hormone, luteinizing hormone and testosterone were purchased from the manufacturers (AccuBind Inc., Lake Forest, USA).

Experimental Design

Chronic toxicity study

The eighty adult male Wistar rats were randomly divided into eight groups of ten Wistar rats as detailed below:

- Group I (DW): served as the control and received 2mL / kg of distilled water daily.
- Group II (Z): received zinc at the dose rate of 50 mg/kg body weight [23].
- Group III (GC): received glyphosate at 14.4 mg / kg body weight (2% concentration in 2 mL distilled water).
- Group IV (G): received 375 mg / kg body weight glyphosate (10% of the LD₅₀) [24].
- Group V (G1): was administered with glyphosate (20% of the LD₅₀), 750 mg / kg body weight as reported by Tizhe [24].
- Group VI (Z + GC): received zinc at 50 mg / kg for 1 hour + glyphosate at 14.4 mg / kg body weight (2% concentration in 2 mL distilled water).
- Group VII (Z + G): received zinc at 50mg/kg for 1 hour + glyphosate 375 mg/Kg (10% of the LD50).
- Group VIII (Z + G1): received zinc at 100mg/kg for 1 hour + glyphosate 750 mg/Kg (20% of the LD50).

The dose regimens were administered per so by gavage once daily for a period of 36 weeks as recommended by OECD [25]. The rats were weighed weekly for appropriate dosing using elec-

tronic digital balance (Hangzhou Gongheng, Electronic Weighing Scale, China).

Determination of semen characteristics

Sperm viability (life and death ratio)

Live and dead sperm cells were distinguished by adding one drop (10 - 15 μ L) of eosin nigrosine stain to one drop of semen at room temperature, for 1-2 min, and smearing the mixture on a clean grease free slide and then examined microscopically using x10 and then x40 objectives to count the percentage of viable and non-viable cells [26]. Viable spermatozoa remained unstained while non-viable spermatozoa stain red. Two hundred (200) cells were counted and the average taken and expressed as percentage (%).

Sperm Motility

This was evaluated by the method of Sonmez [27]. The fluid obtained from the left caudal epididymis was diluted with pipette to 0.5 mL with Tries buffer solution. A slide was placed on light microscope with heater table, an aliquot of this solution was on the slide and percentage motility was evaluated visually at a magnification of x400. Motility estimates were performed from three different fields in each sample. The mean of the three estimates was used as the final motility score. The percentage of sperm motility was calculated using the number of live sperm cells over the total number of sperm cells, both motile and non-motile. The sperm cells that were not moving at all were considered non motile, while the rest, which displayed some movements were considered to be motile.

Epididymis sperm concentration

Spermatozoa in the right epididymis were counted by a modified method of Yokoi and Mayi [28]. Briefly, the epididymis was minced with scissors in 5 mL physiologic saline, placed in a rocker for 10 min and allowed to incubate at room temperature for 2 min. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5g sodium bicarbonate and 1 mL formalin (35%). Total sperm number was determined by using the new improved Neuber's counting chamber (haemocytometre). Approximately, 10 μ L of the diluted sperm suspension was transferred to each counting chamber of the haemocytometre and was allowed to stand for 5 min. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was then focused and the number of spermatozoa counted in five (5) 16-celled squares. The sperm concentration was then calculated and multiplied by five (5) and expressed as [X] x 106 mL⁻¹, where "X" is the number of spermatozoa in a 16-celled square.

Sperm Morphology

The sperm cells were evaluated with the aid of light microscope at x400 magnification. Caudal sperm cells were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin. Five hundred (500) sperm cells from the sample were scored for morphological abnormalities [29]. Briefly, in wet preparations using phase contrast optics, spermatozoa were categorized. In this study, a sperm cell was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, round head and detached head and was expressed as a percentage of morphologically normal sperm [29].

Determination of testicular sialic acid

Semen samples were obtained from the epididymis of the testes after the Wistar rats were sacrificed under light chloroform anaesthesia. The semen samples were allowed to liquefy for 20 min at room temperature and the sialic acid concentrations were determined according to the method outlined by Warren [30].

Determination of concentration of follicle-stimulating hormone, luteinizing hormone and testosterone

The concentrations of serum FSH, LH and testosterone were assayed using their Enzyme Immune Assay (EIA) kits (Monobind Inc. Lake Forest, CA92630, USA).

Histopathological examination

Samples measuring at most 0.5 cm in diameter of testes were taken for histopathological sections preparation and examination after chloroform anaesthesia which was used only at the end of the study so as to prevent pain in the rats. The samples collected were fixed in 10% neutral buffered formalin; they were processed for histopathological assessment using the method outlined by Baker [31] and viewed under light microscope; histochemical features of the testes were also studied by using Sudan black stain after sectioning the 10% buffered formalin fixed samples as outlined by Bancroft and Gamble [32].

Results

Effects of Treatments on Reproductive Parameters

Effects of treatments on sperm concentration

There was a significant increase ($p < 0.05$) in sperm concentration recorded in Z + G1 group as compared to the sperm concentration in G1 group; however, there was relative increase in groups Z + Gc (1%), Z (1.7%) and Z + G (27.6%) when compared to the sperm concentration in DW group. On the other hand, there was relative decrease in the sperm concentration in groups G (7.7%), G1 (16.2%) and Gc (16.6%) when compared to the sperm concentration in DW group as shown on (Table 1).

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Parameters	Groups / Mean ± Sem							
	DW	Z	Gc	G	G1	Z+GC	Z+G	Z+G1
Sperm concentration M/ml	49.4± 0.23	50.2± 6.15	41.2± 4.77	45.6± 2.79	41.4d±2.33	49.9± 4.01	63.0± 3.84	69.3c±1.5
Live sperm (%)	75.7± 3.53	73.5± 7.50	65.50± 10.76	71.3± 2.40	59.3± 13.71	80.0± 0.91	83.5± 5.20	86.7± 2.19
Dead sperm (%)	24.3± 3.53	38.3± 10.71	40.7± 12.5	28.7± 2.4	40.8± 13.7	20.0± 0.9	16.5± 5.20	13.3± 2.19
Normal sperm morphology (%)	86.7± 1.86	81.0± 3.46	76.0± 6.56	74.3± 5.61	66.5c±3.12	76.5± 4.25	78.0± 1.78	83.7± 1.45
Abnormal sperm morphology (%)	13.3± 1.86	15.0± 2.00	29.3± 5.66	22.7± 3.93	33.5c± 3.12	23.5±4.25	22.0± 1.78	16.3± 1.45
Sperm head abnormality (%)	5.3± 0.88	10.0± 3.00	12.3± 1.80	11.0± 1.16	21.0a± 3.34	14.3± 1.89	11.8± 1.32	7.0± 0.58
Sperm mid-piece abnormality (%)	1.0± 0.58	2.7± 0.33	2.8± 1.60	0.67± 0.67	1.0± 0.71	0.0± 0.0	0.5± 0.05	1.3± 0.88
Sperm tail abnormality (%)	7.0± 0.58	8.3± 1.20	12.0± 4.00	9.3± 2.40	11.5± 0.87	9.3± 2.40	8.7± 1.45	8.0± 1.53
Sperm motility (%)	83.0± 0.58	74.3± 6.98	73.3± 5.34	69.0± 2.65	70.0± 5.29	82.0± 4.30	69.3± 2.33	85.0± 1.16
Sperm progressive movement (%)	71.3± 2.19	55.7± 6.57	54.8± 5.07	48.7± 6.89	42.5c± 2.63	51.8± 6.53	32.3b± 5.61	58.0± 4.62
Sperm non-progressive movement (%)	11.7± 2.33	18.7± 3.84	18.5± 2.10	20.3± 4.67	33.5c± 2.63	29.3± 9.26	33.7c± 4.06	27.0± 3.46
Non motile sperm (%)	17.0± 0.58	28.7± 4.10	25.5± 5.39	31.0± 2.65	24.0± 7.07	18.0± 4.30	28.5± 1.50	15.3± 1.20
Sialic acid conc. mg/100 ml	30.3± 1.11	30.2± 5.04	23.6± 3.46	25.3± 4.52	28.6± 3.00	22.5± 1.47	31.4± 2.10	29.8± 0.23

* a = p < 0.001 *b = p < 0.01 *c = p < 0.05 * d = a group used for comparison other than DW.

Table 1: Effects of chronic exposure to distilled water (DW), Zinc at 50 mg/kg, glyphosate at 14.4 mg/kg (GC), glyphosate at 375 mg/kg (G), glyphosate at 750 mg/kg (G1), zinc at 50 mg/kg + glyphosate at 14.4 mg/kg (Z + GC), zinc at 50 mg/kg + glyphosate at 375 mg/kg (Z + G) and zinc at 100 mg/kg + glyphosate at 750 mg/kg (Z + G1) for 36 weeks on reproductive parameters in Wister rats.

Effects of treatments on live sperm count

The result of this study showed no significant difference in the live sperm count between the groups; however, there were relative increase in the live sperm count in groups Z + Gc (5.7%), Z + G (10.3%) and Z + G1 (14.5%) when compared to the live sperm count in DW group. Conversely, there were relative decrease in the live sperm count in groups Z (2.9%), G (5.7%), Gc (13.4%) and G1 (21.7%) when compared to the live sperm count in the DW group as shown on (Table 1).

Effects of treatments on dead sperm count

The result of this study showed no significant difference in the dead sperm count between the groups; however, there were relative decrease in dead sperm count in groups Z + Gc (17.8%), Z + G (32.2%) and Z + G1 (45.2%) when compared to DW group. Conversely, there were relative increase in the dead sperm count in groups G (17.8%), Z (57.5%), Gc (67.2%) and G1 (67.5%) when compared to the dead sperm count in DW group as shown on (Table 1).

Effects of treatments on normal sperm morphology

There was a significant decrease ($p < 0.05$) in the normal sperm concentration in this study in group G1 when compared to that of DW group; however, there were relative decrease in the normal sperm morphology in groups Z + G1 (3.5%), Z (6.5%), Z + G (10%), Z + Gc (11.7%), Gc (12.3%) and G (14.2%) when compared to the normal sperm morphology in DW group as shown on (Table 1).

Effects of treatments on abnormal sperm morphology

The result of this study showed a significant increase ($p < 0.05$) in the abnormal sperm morphology in G1 group when compared to that of DW group; however, there were relative increase in the abnormal sperm morphology in groups Z (12.5%), Z + G1 (22.5%), Z + G (65%), G (70.1%), Z + Gc (76.3%) and Gc (119.4%) when compared to the abnormal sperm morphology recorded in DW group as shown on (Table 1).

Effects of treatments on sperm head abnormality

There was a very highly significant increase ($p < 0.001$) in head abnormality recorded in G1 group as compared to the head abnormality seen in DW group; however, there were relative increase in the head abnormality recorded in groups Z + G1 (31.3%), Z (87.5%), G (106.3%), Z + G (120.3%), Gc (129.7%) and Z + Gc (167.2%) when compared to the sperm head abnormality observed in DW group as shown in (Table 1).

Effects of treatments on mid-piece abnormality

There was no change in the mid-piece abnormality recorded in G1 group when compared to DW group; however, there were relative decrease in the mid-piece abnormality recorded in groups

G (33.3%), Z + G (50%) and Z + Gc (100%) when compared to the mid-piece abnormality seen in DW group. conversely there were relative increase in the mid-piece abnormality in groups Z + G1 (33.3%), Z (166.7%) and Gc (175%) when compared to the mid-piece abnormality recorded in DW group as shown on (Table 1).

Effects of Treatment on Sperm Tail Abnormality

The result of this study showed no significant difference in the tail abnormality between the groups; however, there were relative increase in the tail abnormality observed in groups Z + G1 (14.3%), Z (19%), Z + G (23.8%), Z + Gc (32.1%), G (33.3%), G1 (64.3%) and Gc (71.4%) when compared to DW group as shown on (Table 1).

Effects of Treatment on Sperm Motility

There was no significant difference in sperm motility recorded in this study between the groups; however, there was a relative increase in sperm motility observed in group Z + G1 (2.4%) when compared to the sperm motility recorded in the DW group. On the other hand, there were relative decrease in sperm motility in groups Z + Gc (1.2%), Z (10.5%), Gc (11.8%), G1 (15.7%), Z + G (16.5%) and G (16.9%) when compared to the sperm motility observed in DW group as shown on (Table 1).

Effects of treatments on spermatozoan progressive movement

There was a significant decrease ($p < 0.05$) and a highly significant decrease ($p < 0.01$) in spermatozoan progressive movement in G1 and Z + G groups respectively when compared to the spermatozoan progressive movement observed in DW group; however, there were relative decrease in the spermatozoan progressive movement in groups Z + G1 (18.7%), Z (22%), Gc (23.2%), Z + Gc (27.5%) and G (31.8%) when compared to the spermatozoan progressive movement in DW group as shown on (Table 1).

Effects of treatments on spermatozoan non-progressive movements

There was a significant increase in the spermatozoan non-progressive movement ($p < 0.05$) in G1 and Z + G groups, however, there were relative increase in the spermatozoan non-progressive movement in groups Gc (58.5%), Z (60%), G (74.4%), Z + G1 (131.4%) and Z + Gc (151.3%) when compared to the spermatozoan non-progressive movement in DW group as shown on (Table 1).

Effects of treatments on spermatozoa non-motility

There was no significant difference in the spermatozoan non-motility in this study between the groups; however, there was a relative decrease in the spermatozoan non-motility in Z + G1 (9.8%) group when compared to the spermatozoan non-motility observed in DW group. On the other hand, there was relative increase in the spermatozoan non-motility in groups Z + Gc (5.9%),

G1 (41.2%), Gc (50%), Z + G (67.7%), Z (68.6%) and G (82.4%) when compared to the non-motility observed in the spermatozoa in DW group as shown on (Table 1).

Effects of treatments on testicular sialic acid concentration

There was no significant difference in the sialic acid concentration between the groups; however, there was a relative increase in the sialic acid concentration in Z+G group (3.5%) when compared to the sialic acid concentration in DW group. On the other hand, there were relative decrease in the sialic acid concentration in groups Z (0.4%), Z + G1 (1.8%), G1 (5.7%), G (16.5%), Gc (22.3%) and Z+Gc (25.8%) when compared to DW group as shown on (Table 1).

Effects of treatments on follicle stimulating hormone (FSH) concentration

There was no change observed in the FSH concentration in groups Z, Z + G, Z + G1, Gc and G1 when compared to the FSH concentration in DW group; however, there were relative increase in the FSH concentration in groups G (0.1%) and Z + Gc (0.1%) when compared to the FSH concentration in DW group as shown in (Figure 1).

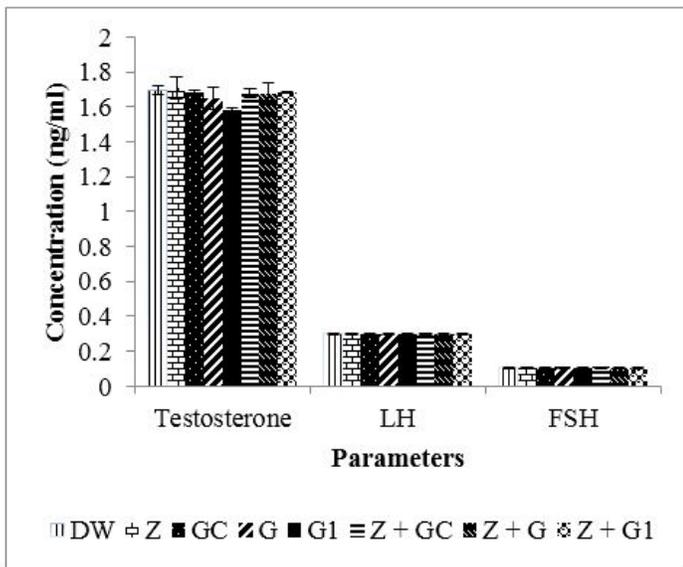


Figure 1: Effects of chronic exposure to distilled water (DW), zinc at 50 mg/kg (Z), glyphosate at 14.4 mg/kg (GC), glyphosate at 375 mg/kg (G), glyphosate at 750 mg/kg (G1), zinc at 50 mg/kg + glyphosate at 14.4 mg/kg (Z + GC), zinc at 50 mg/kg + glyphosate at 375 mg/kg (Z + G) and zinc at 100 mg/kg + glyphosate at 750 mg/kg (Z + G1) for 36 weeks on serum FSH, LH and Testosterone concentration in Wistar rats.

Effects of treatments on Luteinizing Hormone (LH) concentration

There was no change observed in the LH concentration in groups Z, Z + Gc, Z + G and Gc when compared to the LH con-

centration in DW group; however, there were relative increase in LH concentration in groups Z + G1 (0.03%), G (0.03%) and G1 (0.03%) when compared to the LH concentration in DW group as depicted in (Figure 1).

Effects of treatments on testosterone concentration

There was no significant difference in the testosterone concentration between the groups; however, there was a relative increase in the testosterone concentration in Z (0.7%) group when compared to the testosterone concentration in DW group. Conversely, there was a relative decrease in the testosterone concentration in groups Z + G1 (0.6%), Gc (0.8%), Z + Gc (1.1%), Z + G (1.2%), G (2.6%) and G1 (7%) when compared to the testosterone concentration in DW group as shown in (Figure 1).

Histopathological findings

The testes of rats in group I (DW) showed no observable lesion as represented on (Plate Ia).

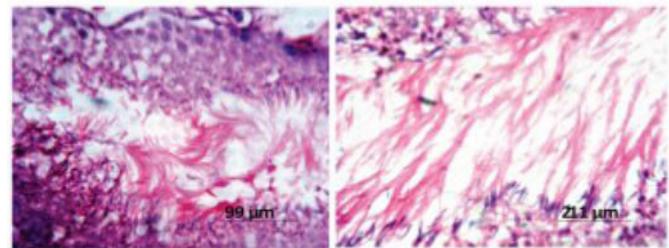


Plate Ia: Photomicrograph of testis of rat administered distilled water (DW) for 36 weeks showing no observable microscopic lesions (H & E).

Similarly, the testes of rats in groups II (Z), VI (Z + Gc), VII (Z + G) and VIII (Z + G1) showed no observable lesion similar to those of group I (DW), however, vacuolations in the seminiferous tubules, degeneration of spermatogenic cells and decreased number of spermatids in the seminiferous tubules were observed in the testes of rats in group III (Gc) as shown on (Plate IIa).

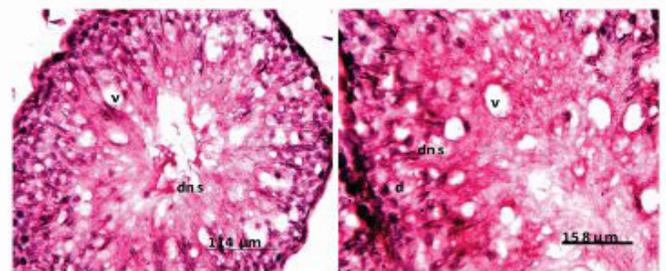


Plate IIa: Photomicrograph of testis of rat administered glyphosate (Gc) at 14.4 mg/kg for 36 weeks showing vacuolations in the seminiferous tubules (v), decreased number of spermatids (dns) and degeneration of spermatogenic cells (d). (H & E).

The testes of rats in group IV (G) revealed vacuolations in the seminiferous tubules and degeneration of the spermatogenic cells as depicted on (Plate IIIa).

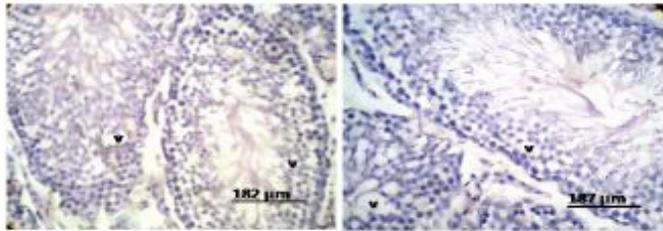


Plate IIIa: Photomicrograph of testis of rat administered glyphosate (G) at 375 mg/kg for 36 weeks showing vacuolations in seminiferous tubules (v). (H & E).

Vacuillations in the seminiferous tubules, degeneration of spermatogenic cells, fragmented and coiled spermatids which were detached from the basement membrane were observed in the testes of rats in group V (G1) as shown on plate IVa.

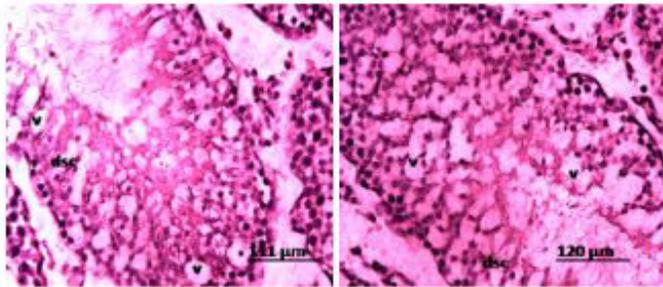


Plate IVa: Photomicrograph of testis of rat administered glyphosate (G₁) at 750 mg/kg for 36 weeks showing vacuolation in the seminiferous tubule (v) and degeneration of spermatogenic cells (dsc). (H & E).

Histochemical investigation of testes of Wistar rats in this study using Sudan Black (SB) stain showed normal histoarchitecture in the testes of Wistar rats in groups DW, Z, Z + Gc, Z + G and Z + G1⁻ as shown on (Plate Ib)

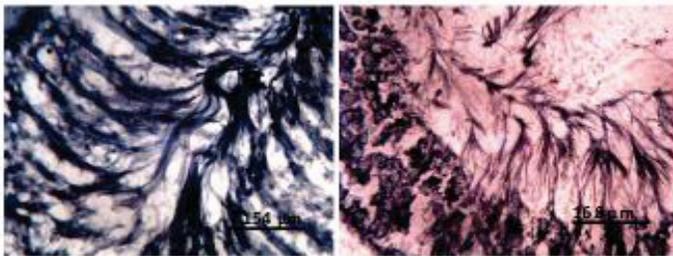


Plate Ib: Photomicrograph of testis of rat administered distilled water (DW) for 36 weeks showing no observable microscopic lesions (SB).

while the testes of Wistar rats in groups Gc, G and G1 revealed vacuolations in the seminiferous tubules as represented on (Plate I Ib, IIIb and IVb) respectively.

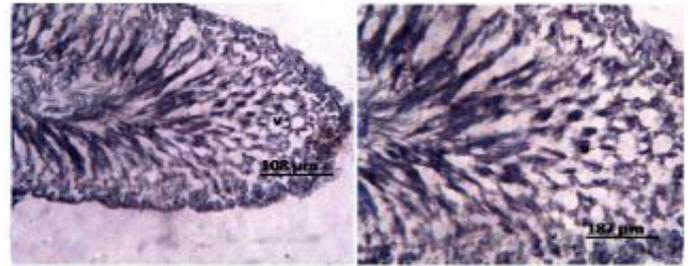


Plate IIIb: Photomicrograph of testis of rat administered glyphosate (G) at 375 mg/kg for 36 weeks showing vacuolations in seminiferous tubules (v). (SB).

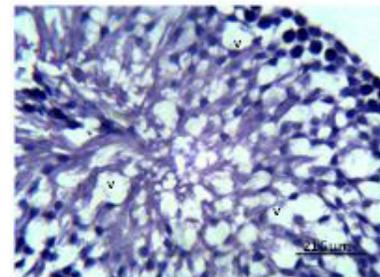


Plate IIIb Photomicrograph of testis of rat administered glyphosate (G₁) at 14.4 mg/kg for 36 weeks showing vacuolations in the seminiferous tubules (v). (SB).

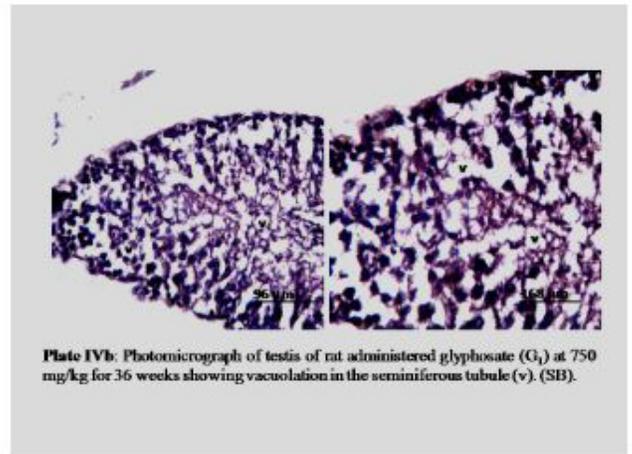


Plate IVb: Photomicrograph of testis of rat administered glyphosate (G₁) at 750 mg/kg for 36 weeks showing vacuolation in the seminiferous tubule (v). (SB).

Discussion

Chronic glyphosate exposure in this study showed decrease in sperm concentration in all the groups treated glyphosate alone which might be as a result of increased generation of the Reactive Oxygen Species (ROS) in the testes which might have impaired

spermatogenesis in the exposed rats. The induction of oxidative stress in testicular cells by Roundup®, a glyphosate based herbicide, was said to be evident by decreased glutathione reductase levels accompanied by increased Thiobar Bituric Acid Reactive Substance (TBARS) levels by Roundup® in rat's testes thus linking ROS over-generation and oxidative damage [33]. Spermatozoa on their own are highly susceptible to oxidative damage by excessive ROS due to high concentration of Poly Unsaturated Fatty Acid (PUFA) within their plasma membrane [34]. Reduction in epididymis sperm count following exposure to organophosphate, chlorpyrifos, was reported by earlier researchers to be caused by low levels of scavenging enzymes and glutathione as well as high production of free radicals, resulting from mitochondrial respiration and deficient DNA repair mechanisms and thus providing unfavorable condition for spermatogenesis in the seminiferous tubules [22,35,36]. The slight increase in the sperm concentration in the zinc group, Z, might underscore the antioxidant effect of zinc as evident by increased sperm concentration in the group when compared to the sperm concentration recorded in DW group. Similarly, pretreatment with zinc in zinc supplemented groups, Z+Gc, Z+G and Z+G1 caused apparent increase in sperm concentration in the said groups when compared to the sperm concentration in the DW group and a significant increase in the sperm concentration was obtained in Z + G1 group when compared to that of G1 group probably mediated by the antioxidant effect of zinc which might have mitigated the probable impaired spermatogenesis observed in the G1 group sequel to glyphosate exposure. Zinc had been reported to play an important role in maintenance of structure and function of biological membrane by scavenging free radicals [37]. Protective role of antioxidant (vitamin C) in increasing sperm count following pretreatment in chronically chlorpyrifos-exposed rats had been reported by Shittu [38].

Live sperm count in this study decreased in the glyphosate exposed groups when compared to the live sperm count in DW group. The decreased live sperm count associated with chronic glyphosate exposure in this study might be due to the level of the oxidative damage in the testes of the exposed rats. Razi [39] in a sub-chronic study on glyphosate exposure in rats reported decreased sperm viability alongside other alterations in reproductive parameters in the glyphosate exposed rats. Zinc administration caused a relative decrease in the live sperm count which might be associated with slight prooxidant effect of the zinc on the sperm viability in the rats in the Z group. Prooxidant effect of zinc was reported earlier by Abdallah and Samman [40]. On the other hand, pretreatment with zinc in the zinc supplemented groups revealed apparent protective effect of zinc in the mentioned groups as evident by the apparently higher live sperm count in all the zinc supplemented groups when compared to the live sperm count in the DW group possibly due to the antioxidant role of zinc in the zinc supplemented groups. Zinc plays a structural role in the maintenance of the integrity of Cu - Zn superoxide dismutase as a cofac-

tor [41] and it is also known to regulate glutathione that is vital to cellular antioxidant defense [42].

The dead sperm count in this study increased in all the glyphosate exposed groups and the increase in the percentage of dead sperm count might be as a result of high level of oxidative damage caused by oxidative stress in the testes of the exposed rats. Increased dead sperm count has been reported in a sub-chronic toxicity study on reproduction in rats by Razi [39]. A relatively high dead sperm count was also recorded in the rats administered zinc alone in Z group possibly due to prooxidant effect of the zinc in the rats in that group as reported by Abdallah and Samman [40] since the rats were not exposed to any environmental toxicant. Zinc supplementation in the zinc pretreated rats, however, ameliorated the lethal oxidative damage of the glyphosate on the sperm by apparently reducing the dead sperm count to levels below that of the DW group in all the rats in zinc supplemented groups which were likely brought about by the antioxidant effect of zinc. Apart from its direct antioxidant role by occupying iron and copper binding sites on lipids, proteins and DNA as reported by Prasad and Kucuk [43], zinc also plays a structural role in the maintenance of the integrity of Cu - Zn superoxide dismutase as a cofactor as well as maintaining the structure and function of biological membranes [37,41]. Normal sperm morphology in this study decreased in a dose-dependent fashion in the glyphosate exposed groups with a significant decrease observed in the highest glyphosate exposed group which might have been occasioned by increased generation of ROS causing oxidative damage to the normal architecture of the spermatozoa. Morphologically abnormal spermatozoa, precursor germ cells, leucocytes and at last degenerated cells in spermatogenesis series are the components of the male genital system capable of generating Reactive Oxygen Species (ROS) as reported by Chapin [44]. A dose-dependent reduction in morphologically normal spermatozoa which was said to be the possible cause of the decreased sperm concentration was also recorded in a dose-dependent fashion in chlorpyrifos toxicity study as earlier reported by Olorunshola [45]. A relative decrease in the normal sperm morphology recorded in the group administered with zinc alone, Z group, might be as a result of prooxidant effect of zinc in the absence of oxidative stress in the rats in Z group as reported by Tizhe [46]. Zinc pretreatment in the zinc supplemented groups, showed variable ameliorative effect by increasing the percentages of the normal spermatozoa in the zinc supplemented groups to near normal when compared to those of the glyphosate exposed groups. The ameliorative effect of antioxidant on reduction in the sperm morphology in chlorpyrifos toxicity study was earlier reported by Olorunshola [45].

Abnormal sperm morphology in this study was shown to significantly increase in G1 group which is the highest glyphosate exposed group and apparent increase in the abnormal sperm morphology were recorded in Gc and G groups and the increased ab-

normality observed in all the glyphosate exposed groups might be attributed to the oxidative damage caused by probable increased generation of free radicals in the testes. Razi [39] documented elevated abnormal sperm content with different characteristics in glyphosate exposed groups which they attributed to the probable major role of imbalanced oxidative stress in generating various disorders. Zinc administration in Z group caused relative increase in the abnormal sperm morphology observed in the rats in Z group possibly as a result of prooxidant effect of zinc in the rats since they were not exposed to any environmental toxicant. Prooxidant effect of zinc was earlier documented by Abdallah and Samman [40]. Pretreatment with zinc in groups which were supplemented with zinc showed ameliorative effect by decreasing the levels of the abnormal sperm morphology in the said groups when compared to the glyphosate exposed groups which might have been caused by the protective role of zinc in the maintenance of morphology of the sperm cells. Zinc was reported to protect the testes against degenerative changes [47]. Bettger and O'Dell [37] reported the role of zinc in maintenance of structure and function of biological membranes.

Sperm head abnormality in this study was shown to be apparently increased in the glyphosate-exposed groups when compared to DW group which might possibly be sequel to increased level of oxidative damage to the sperm cells. Increased abnormal sperm content with different characteristics such as elongated head, pyriform head, bent head, cytoplasmic droplets and degenerated germ cells were reported by Raji [39]. Zinc administration in Z group caused apparent increase in the sperm head abnormality possibly mediated by the pro-oxidant effect of zinc as reported by earlier researchers [40,46]. Zinc supplementation in Z + Gc and Z + G did not ameliorate the increased abnormal sperm head recorded in the said groups, however, an apparent ameliorative effect was recorded in Z + G1 group which was the highest glyphosate exposed group that was supplemented with zinc at high concentration; it is therefore, possible that the increased sperm head abnormality recorded in Z + Gc and Z + G which were not ameliorated might be ameliorated with increased zinc supplementation similar to that used for Z + G1 group. The role of zinc as antioxidant to protect the cell membrane and nuclear chromatin of spermatozoa was earlier reported by Chvapil [48]. Various changes were observed in sperm mid-piece abnormality following glyphosate exposure in this study ranging from apparent increase in the sperm mid-piece abnormality in Gc group to a decrease in the mid-piece abnormality in G group and no noticeable difference in the sperm mid-piece abnormality in G1 when compared to DW group. The changes recorded in the sperm mid-piece abnormality in this study suggested that most of the abnormal changes seen were associated with the head and the tail of the sperm cells but the reason for the varying changes recorded is not known for certain especially considering the similar pattern of changes observed in the sperm head abnormality and the sperm tail abnormality in this study. Zinc

administration in Z group caused apparent increase in the sperm mid-piece abnormality similar to that seen in the sperm head abnormality in this study following zinc administration in Z group probably associated with pro-oxidant effect of zinc in the rats in the group. Pro-oxidation in animals following zinc administration was reported in earlier study by Abdallah and Samman [40]. Zinc supplementation in Z + Gc and Z + G groups effectively ameliorated the sperm mid - piece abnormality in the rats in those groups but ameliorative effect was not observed in the sperm mid - piece abnormality recorded in Z + G1 group and the reason for that is not clear but might be as a result of the highest level of oxidative damage in the group.

Zinc can counteract oxidation by binding suphydryl groups in proteins and by occupying binding sites for iron and copper in lipids, proteins and DNA [44,49] and to substantiate these antioxidant effect of zinc, evidence was found for oxidative damage of proteins lipids and DNA in zinc-deficient rat and mice [50,51].

Sperm tail abnormality in this study increased in all the glyphosate exposed groups, likely due to increased level of oxidative damage in the sperm cells of the rats in the aforementioned groups. Sperm abnormalities were documented in earlier studies on organophosphates toxicity in rats [39,45]. Zinc administration in Z group in this study caused relative increase in the sperm tail abnormality when compared to DW group which might be as a result of the pro-oxidant effect of the zinc similar to those of head and mid - piece abnormality in the sperm cells observed in this study. Abdallah and Samman [40] have documented the prooxidant effect of zinc. Zinc supplementation in the zinc pretreated groups caused ameliorative effect by the decreased sperm tail abnormality to near normal probably due to the antioxidant role of zinc. Zinc salts have been shown to protect against oxidative damage and glutathione depletion in mice [51]. Decreased sperm motility was recorded in glyphosate - exposed groups, in this study which might be attributed to the possible oxidative damage in the testes caused by glyphosate exposure in the affected rats. Choudhary [52] documented that sperm motility is affected by altered enzymatic activities of oxidative phosphorylation. Full ATP pool is essential for normal spermatozoan movement and a slight deprivation of ATP leads to reduction in sperm motility which may cause infertility [53]. Another factor which may cause decrease in sperm motility may be androgen deprivation effect of the pesticide [52]. A positive correlation between testosterone and motility and fertilizing capacity of the spermatozoa has been reported and the compounds of seminal vesicle secretion also act as energy source for sperm motility [54]. Lipid peroxidation, had been documented to destroy the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility [55-57]. Decreased sperm motility in organophosphates toxicity in rats had been documented by earlier researchers [39,45]. Decrease in sperm motility has been documented to be caused principally by defective spermatozoa [58].

Zinc administration in Z group caused decreased sperm motility possibly due to pro-oxidant role of zinc in the absence of oxidative stress in the rats. Zinc pretreatment in zinc supplemented groups, apparently increased the rate of motility in the sperm cells in the rats in the said groups. Decreased sperm motility was documented to be ameliorated following the use of an antioxidant (Vitamin C) in a previous study by Olorunshola [45].

Decrease in sperm progressive movement were recorded in all the glyphosate exposed groups, which was more pronounced in G1 group where a significant decrease in the sperm progressive movement was recorded. The decline in the sperm progressive movement observed following glyphosate exposure in this study might be associated with some oxidative damage in the viability of the sperm and/or the testosterone level in the rats. Decrease in sperm motility has been documented to be caused principally by defective spermatozoa [58]. The epididymis spermatozoa are highly dependent on testosterone and epididymis protein for their final maturation and development of progressive motility and fertilizing capacity [59]. Zinc administration in Z group showed apparent decrease in the sperm progressive movement which might be associated with pro-oxidant effect of zinc in the rats since they were not under oxidative stress. Proxidant effect of zinc was documented in earlier studies [40,60]. Zinc supplementation in Z + Gc and Z + G did not show any protective effect but instead it further decreased the sperm progressive movement in the aforementioned groups, however, zinc supplementation in Z + G1 group has greatly ameliorated the sperm progressive movement in the rats in that group, possibly due to the high dose of zinc used for supplementation in that group. Olorunshola [45] has documented the ameliorative role of an antioxidant, vitamin C, in decreased sperm motility observed in their study on chlorpyrifos toxicity in rats.

Non-progressive movement in spermatozoa in this study apparently increased in a dose dependent fashion in the different doses of glyphosate used for the study and the decrease was statistically significant in G1 group which was the highest glyphosate exposed group. The changes recorded might be due to the increased level of oxidative stress in the testes or due to decrease in the testosterone level in the rats. Vawda and Davies [59] documented that epididymis spermatozoa are greatly dependent on testosterone and epididymis protein for their final maturation and development of progressive motility and fertilizing capacity. The increased non-progressive motility recorded following glyphosate exposure in this study might also be as a result of increased deprivation of ATP to the spermatozoa since full ATP was said to be crucial for the normal spermatozoon movement and any slight deprivation of ATP leads to reduction in spermatozoon movement and may cause infertility (Bedford, 1983). Zinc administration in Z group caused apparent increase in the non - progressive movement of sperm in this study probably mediated by the pro-oxidant effect of zinc since the rats in Z group were not under oxidative stress. Pro-oxidant effect associated with zinc was documented in earlier

studies [40,46,60]. Zinc supplementation in zinc pretreated groups did not show ameliorative effect but instead further increased the sperm non - progressive movement in a similar pattern of negative effect seen in the sperm progressive movement in this study and the non-progressive sperm movement in this study is significant in Z+G group, however, ameliorative effect was apparently seen in Z + G1 similar to that recorded for sperm progressive movement in this study probably caused by the antioxidant effect of zinc in the rats since the zinc was in high dose in that group and might therefore, account for the ameliorative effective recorded in Z+G1 group which was not seen in Z+Gc and Z+G group. Vitamin C as an antioxidant was used in a previous study by Olorunshola [45] to mitigate various sperm abnormal changes recorded in the study.

Glyphosate exposure in a chronic study in this research has been shown to cause a dose dependent apparent increase in the number of non-motile sperm cells observed in the glyphosate exposed groups, probably caused by oxidative damage in the testis or alteration in the testosterone levels in the Wistar rats. Lipid peroxidation was said to destroy lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility [55-57]. Complete ATP pool is necessary for normal spermatozoon movement and slight deprivation of ATP leads to reduction in spermatozoon movement which may cause infertility (Bedford) [53]. Zinc administration in Z group caused an apparent increase in the number of non-motile sperm cells observed in the Z group possibly due to the pro-oxidant effect of zinc as earlier reported by Abdallah and Samman [40]. Zinc supplementation in zinc pretreated groups ameliorated the detrimental effect of the glyphosate by reducing the number of the non-motile sperm cells in the rats pretreated with zinc in the said groups probably due to the antioxidant role of zinc as documented by Olorunshola [45] who used vitamin C as an antioxidant to ameliorate the adverse effect of chlorpyrifos in the spermatozoa of the chlorpyrifos - exposed rats.

Testicular sialic acid concentration decreased in all the glyphosate exposed groups, in this study likely due to the increased oxidative stress in the testes of the rats in the aforementioned groups. In a similar trend, Choudhary [52] reported a significant decline in the contents of sialic acid among other reproductive parameters they studied following exposure of male rats to malathion. Zinc administration in Z group did not have negative effect on the testicular sialic acid concentration in this study, possibly because of the antioxidant effect of the zinc on the testicular sialic acid and zinc supplementation in the zinc pretreated groups showed a noticeable ameliorative effect by restoring the sialic acid concentration to near normal in the zinc supplemented groups. Several studies have demonstrated the protective role of zinc on organophosphates compounds toxicity [22,24,46,61-63].

In this study, however, chronic exposure to glyphosate in male Wistar rats caused a dose- dependent decrease in testosterone concentration in the glyphosate exposed groups which might be

linked to the possible oxidative damage to the testes of the glyphosate exposed rats. Most earlier researches on organophosphate compounds recorded decrease in testosterone concentration which was said to be perhaps as a result of the inhibitory effect of organophosphates on the secretion of pituitary gonadotropins (FSH and LH), which are involved in testosterone biosynthesis, direct damage to the leading cells or inhibition of testosterone metabolism or testosterone synthesis [23,45,64-66]. Conversely, chronic exposure to sub-lethal concentration of a glyphosate-based herbicide (3.6mg/L) in female *Jundia* showed increased pattern of testosterone secretion which was similar to the progression of the vitellogenesis. Zinc administration in the Z group caused relative increase in the serum testosterone concentration which might underscore an antioxidant role of zinc in increasing serum testosterone concentration. Zinc had been reported to protect sulphhydryl group against oxidation thereby preventing protein from oxidation, hence stabilizing the cellular thiol pools [67]. On the other hand, pretreatment with zinc as an antioxidant restored the testosterone concentration to near normal in Z+G and Z+G1 groups thus, underscoring the possible involvement of oxidative mechanism in the pathology of glyphosate in rats. Previous studies on the use of antioxidants to ameliorate the decreased testosterone levels following exposure to organophosphates have been documented [23,45]. Conversely, pretreatment with zinc in Z+ Gc group did not ameliorate the relative decrease in serum testosterone level and the reason for that is not known especially considering the relative increase in the level of serum testosterone in Z group and the apparent ameliorative effect recorded following pretreatment with zinc in Z+G and Z+G1 groups.

Chronic glyphosate exposure in this study showed no difference in the FSH concentration between the glyphosate treated groups Gc and G1 when compared to the DW group, perhaps due to minimal effect or no toxic effect of the glyphosate on FSH because the only glyphosate exposed group that showed a negligible difference is G group which showed a slight increase in the FSH concentration when compared to DW group and that is negligible to be linked only to glyphosate because minor difference in concentration can be seen even within normal animals. The result of this study is at variance with most studies on organophosphate compounds effect on FSH concentration in animals who documented that organophosphates cause decrease in the serum levels of FSH probably due to the ability of the organophosphate compounds to suppress the gene involved in gonadotrophins synthesis or interfere with steroidogenesis [8,23,68]. Zinc administration in Z group also did not cause any noticeable change in the concentration of serum FSH in the rats in that group likely because zinc as an antioxidant has no toxic effect on FSH concentration in animals even on long term exposure. On the other hand, pretreatment with zinc in Z + G and Z + G1 groups also caused no noticeable difference in the serum FSH concentration when compared to DW and those of glyphosate exposed rats alone, however, pretreatment

with zinc in Z + Gc group also caused a negligible increase in FSH concentration. Zinc had been reported to play an important role in the structure and function of biological membranes [37].

The result of this study showed a very negligible increase in LH concentration in glyphosate exposed groups G and G1 and no difference in the LH concentration was recorded in Gc group when compared to the serum LH concentration in DW group and that might be due to the low glyphosate exposure in that group. This result suggests that chronic glyphosate exposure has little or no pathologic and/or toxic effect on serum LH concentration which differs from most previous studies on the serum LH concentration in animal's sequel to organophosphate exposure who documented decreased serum LH concentration which they attributed to be likely due to their effects on Hypothalamo-pituitary endocrine function [8,35,69-71]. In tandem with the finding of this study, increase in serum level of LH following organophosphate exposure had been documented and were said to be detrimental to the germinal cells of the testes and hence capable of disrupting spermatogenesis. Zinc administration in the Z group did not cause any change in the serum level of LH when compared to DW group and pretreatment with zinc in Z + Gc and Z + G also did not cause any change in the serum concentration of LH when compared to DW, however, pretreatment with zinc in Z + G1 group caused a negligible increase in the serum level of LH in Z + G1 group similar to that recorded in G and G1 groups and the reason for that is not known for certain but might be due to the high exposure to the glyphosate in G1 and therefore difficult to mitigate the increase in the serum concentration of the LH even when high zinc supplementation was used. The antioxidant role of zinc in organophosphate toxicity had well been documented in previous studies [23,46,61-63].

Histomorphological examination of testes in the study showed vacuolation in the seminiferous tubules, degeneration of spermatogenic cells, fragmented and coiled spermatids with H & E stains, and when stained with Sudan black due to the presence of vacuoles observed when stained with H & E, the sections were still found to have vacuolations in the seminiferous tubules which are most likely fat vacuoles and that might have deleterious effect on spermatogenesis in the glyphosate exposed groups where they were observed. Similar to this finding, severe degeneration in seminiferous tubules and dissociation of germinal cells and arrested spermatocytogenesis, spermatogenesis and spermatogenesis with severe depletion of seminiferous tubules which was accompanied by huge infiltration of inflammatory cells were documented in diazinon exposed rats [72]. There are several independent studies which indicated that following severe inflammation, elevated oxidative stress causes apoptosis in spermatogenesis series characterized by remarkable cellular depletion in seminiferous tubules [73,74]. Gradual decrease in paired testicular weight and seminiferous tubular diameter in association with progressive degeneration

tive changes in seminiferous epithelial cells were found to occur in relation to various doses and durations of pesticide treatment [75]. Zinc administration in Z group and zinc supplementation in zinc pretreated groups showed no observable microscopic lesions in the testes of the rats in those groups, probably due to the ameliorative effect of zinc as an antioxidant which ameliorated the histopathological changes in the testes of rats in the zinc pretreated groups as evident following examination of both the H & E and Sudan black-stained sections. Antioxidant mineral, selenium, had been documented to mitigate the detrimental effects of diazinon in the seminiferous tubules of diazinon exposed rats [72].

Note: There is no conflict of interest among the authors

Contributor ship Statement

Dr. Uchendu Chidiebere, department of Veterinary Pharmacology, Physiology and Biochemistry helped us with the data analysis after the research work. Prof. N.D.G Ibrahim, Prof. M.Y Fatihu, Prof. S.F Ambali and Prof. I.O Igbokwe were very instrumental in the design, supervision and editing the manuscript. I.J. Gosomji Dr. U. Delia and DR. E.V Tizhe contributed in the design, literature review, carrying out the research work and wrote the manuscript for publication.

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