Original Article

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Distinguishing Features of Glyphosate on the Behaviour, Body, Testis and Epididymis of Wistar Rats

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Abstract

There is a concerned growing need to increase agricultural products to meet up with growing world consumption using chemicals. Glyphosate (an herbicide) falls into the group of chemicals with perceived infertility. This study investigated the distinguishing features of glyphosate on the behaviour, body, testis and epididymis of Wistar rats. Fifteen adult male Wistar rats were randomly assigned into three groups. Rats in group A (control) received water; group B (low dose) and group C (high dose) received 400mg and 2000mg of glyphosate/kg body weight/day respectively. Experimental period lasted 60days before sacrificing. Excised testes and epididymis were fixed in Bouin's fluid; and subjected to histological and morphometric analyses. Statistical Package for Social Scientists (version 21) was employed for analysis of collated data. The level of statistical significance was set at $p < 0.05$. Varying degrees of physical changes in groups B and C rats were observed. Those in group C further suffered weight loss, shedding of furs, agitation and loose stools. Glyphosate showed decreasing number of spermatozoa within the reproductive system of groups B and C rats. Dose-dependent toxic effects of glyphosate on the testes and epididymides with subfertility was established probably as illustrated on the histology. Glyphosate appeared to have penetrated blood brain barrier with resultant behavioural changes among treated rats. Anti-fertility effect of glyphosate was linked to great reduction of spermatozoa density and disruption of histological characteristics of male reproductive tract. Nevertheless, the use of glyphosate should be genuinely guided or discouraged as applied in some developed countries.

Keywords: Glyphosate; Toxicity; Histology; Male Infertility.

Introduction

In today's world, there is a concerned growing need to increase agricultural products to meet up with growing world consumption. These agricultural products are daily utilized in food, textile, cosmetic, pharmaceutical and wooden industries. For these usages, numerous compounds (fertilizers, pesticides and herbicides) are being added to boost and improve agricultural output [1].

These various uses have posed a dilemma for physicians dedicated to preserving life and improving reproductive health and for food producers employing all forms of techniques to control weeds and pests. Glyphosate falls into the group of chemicals or medications affecting spermatogenesis such as cancer chemotherapy, anabolic steroids, *Cimetidine* and *Spironolactone*; those that decrease follicle stimulating hormone (FSH) levels such as *Phenytoin*; and those that decrease sperm motility such as *Sulfasalazine* and *Nitrofurantoin* [2]. Glyphosate was believed to be relatively non-toxic to mammals [3]. Preposterous verdict of acceptable risks for glyphosate was championed by the government of Germany. Germany played the European Union Rapporteur Member State and submitted their glyphosate renewal assessment report to the European Food Safety Authority (EFSA), recommending formal approval of glyphosate for use in Europe with increase in the acceptable daily intake [4]. Notwithstanding, the recommendation from Germany was alleged to be scandalous following overwhelming evidence of environmental pollution leading to banning of glyphosate in some countries. These countries include: Brazil, Denmark, El Salvador, France, Netherlands and Sri Lanka [5].

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Male infertility has varying causative factors; among these are hormonal imbalance, exposure to spermicidal agents, physical distress and psychological/behavioural problems. It is a fact: the abuse of anabolic steroids is common in sports and entertainment industries among participants to enhance performance. The hormonal imbalance has impact on the pituitary-gonadal axis and its feedback mechanisms. Endocrinologically, the gonadal and sexual functions are mediated by the hypothalamic-pituitary-gonadal axis, a closed-loop system with feedback control from the testicles [6]. In the absence of any biochemical or hormonal derangement, testicular biopsy is indicated to investigate azoospermic males with a normal-sized testis to assess tubular obstruction; and for further investigation of idiopathic infertility [7].

Glyphosate ($C_3H_8NO_5P$, *N-Phosphonomethylglycine*) is a non-specific world's most widely used herbicide in agriculture. As herbicide, it is, most times, sprayed on plants with relatively high levels permeating as residues in food products, water and animal feeds apart from human exposure [8]. Contamination frequently occurs when rain falls directly after application of glyphosate and through flood to the rivers and lagoons [9]. *Detailed mechanism of action of glyphosate in animal cells had been identified* [5].

Glyphosate increases cytosolic calcium (Ca^{2+}) concentration by opening Ca^{2+} channels, thereby permitting Ca^{2+} into the cells with resultant Ca^{2+} overload and cell death [10,11]. In an experiment, glyphosate toxicity was due to Ca^{2+} overload with resultant cell signalling fault and a stress response in defence against depleted antioxidant, thereby, contributing to the death of Sertoli cells hence infertility [12]. Oxidative stress causes the influx of Ca^{2+} into the cytosol and the organelles, most especially, mitochondria and to nuclei thereby quickens the disruption of normal oxidative metabolism through programmed apoptotic or inflammatory necrotic cell death [13,14].

Within the nucleus, Ca^{2+} modulates gene transcription and nucleases that regulate apoptosis (genetically programmed cell death) that involves fragmentation of deoxyribonucleic acid (DNA) [15].

This study seeks to contribute to the fight against the increasing prevalence of male infertility following the consumption of agricultural products that have absorbed glyphosate and other related chemicals from anatomic point of view using adult male Wistar rats (*Rattus norvegicus*). Specific objectives: assessment of behavioural and weight changes; and determination of the effects of glyphosate on the histology of testis and epididymis of Wistar rats.

Limitation of Study

Only light microscope was available for the histomorphological analysis.

Materials and Methods

This study was approved and carried out according to the international standard adopted by the Department of Anatomy, University of Benin, Benin-City, Nigeria. The adult male Wistar rats used for this study weighed 200g and above. A pilot study earlier conducted for a period of two week on 6 adult male Wistar rats divided into three groups of 2 rats in each: A 'Control' gavaged with only distilled water; B 'Low dose' gavaged with 400mg of glyphosate/kg/day; C 'High dose' gavaged with 2000mg of glyphosate/kg/day.

The LD_{50} of glyphosate is above 4000mg/kg body weight/day and the safest tolerant dose to be administered was taken as half: 2000 mg/kg/day [16-18]. The 6 rats were gavaged for 2 weeks. One of the rats in high dose group died, perhaps, due to the metabolic effect of glyphosate on its systems. The main study comprised fifteen rats which were divided at random as well into 3 groups A, B and C of 5 rats in each group. Their weights were recorded at the beginning of the experiment, at weekly interval, and at the point of sacrifice. All the rats were gavaged for 60 days based on their spermatogenic cycle [19]. The rats were sacrificed at day 61.

Each rat for sacrificing was anaesthetized with Chloroform (*Tetrachloromethane*; CCL_4) in cotton wool in an enclosure for 2-3minutes. Thereafter, the rat was laid supine on a dissecting wooden table and pinned. Using a lower abdominal midline approach, the ductus deferens was quickly ligated at the proximal and distal ends to contain semen for sperm analysis. The pelvic cavity was explored to harvest the testes and epididymides and the tissues were preserved in Bouin's solution after weighing. Testicles and epididymides were processed for light microscopy. Morphometric study of testis and epididymis was done simply by measuring the tubular and luminal diameters with metre rule and evaluated the ratio. The data were collated and entered into Statistical Package for Social Scientists (SPSS version 21) software for t-test, and test of significance ($p < 0.05$). Standard error of the mean (SEM) was applied to assess the effect of random changes of the body weight: the higher the value the higher the degree of random changes [23]. Results were represented in words, tables and figures using Microsoft office software.

Results

The results showed varying degrees of physical and behavioural changes in groups B and C rats when compared to control rats. The male rats in group B had increased appetite and weight gain. Conversely, treated rats in group C had loss of appetite, loose stools, shedding of furs, weight loss and agitation. Mean of different parameters were statistically-significant as shown in the table. Mean body weight of rats was decreasing from control group A to high dose group C. The degree of random changes of body weight was highest among rats in group A with the highest SEM value and least in group B. The body weight was increasing among the rats in groups A and B by 1.8g and 1.0g respectively. Contrarily the value was decreasing by 1.1g in group C rats. Average testicular weights were statistically-significantly decreasing with increasing dose of glyphosate.

The slides for control group A rats showed normal histological features of the testes (Figures 1 and 2) and epididymides (Figures 7 and 8). Nevertheless, among the treated groups, there was evidence of reduced spermatozoa population in the testes and epididymides as evidenced by the reduced number of tufty tails of spermatozoa in the lumina of the seminiferous tubules of the testes and the increased spaces between the epithelial linings of the epididymides and the contained spermatozoa in the lumina (Figures 3, 4, 5, 6, 9, 10, 11, 12). There were reducing average luminal-tubular ratio in the treated groups.

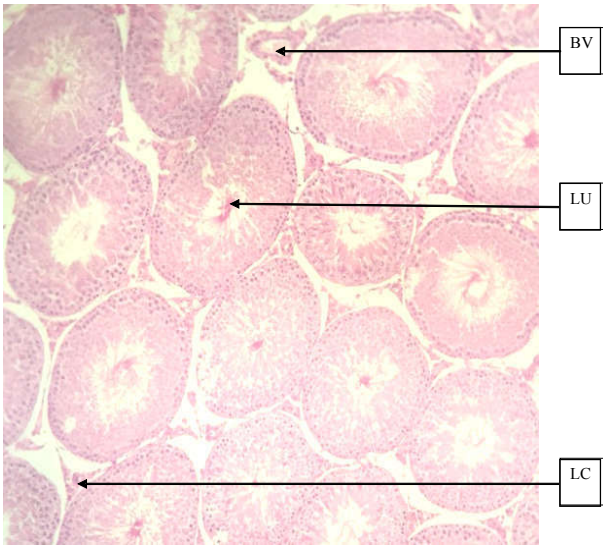

Fig. 1: Photomicrograph of cross section of testes of Group A (Control) male Wistar rats (x100; H & E) showing normal lumen of the seminiferous tubule (LU) densely packed with tufty tails of spermatozoa , blood vessel (BV), interstitial space containing interstitial cells of Leydig (LC). The average luminal-tubular ratio is 1:3.

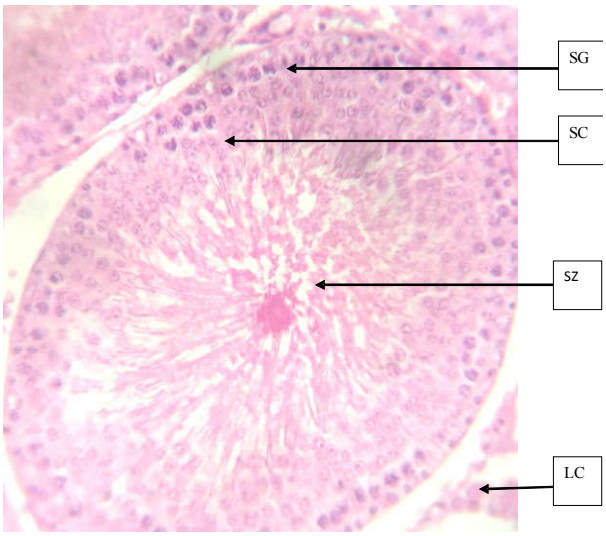

Fig. 2: Photomicrograph of cross section of testes of Group A (Control) male Wistar rat (x400; H & E) showing normal cells of the spermatogenic series; spermatogonia (SG), spermatocytes (SC), lumen of the seminiferous tubules containing densely packed tufty tails of spermatozoa(SZ) and interstitial space containing interstitial cells of Leydig (LC). The luminal-tubular ratio is 1:2.6.

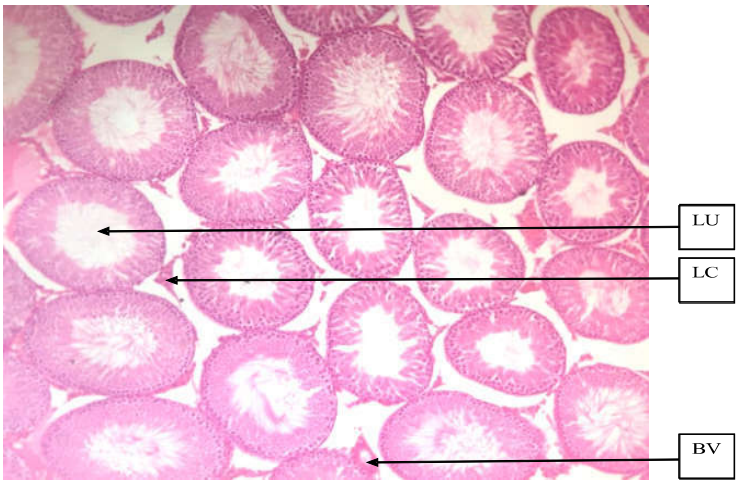

Fig. 3: Photomicrograph of cross section of testes of Group B (400mg/kg body weight/ day of glyphosate) male Wistar rat (x100; H & E) showing cells of the spermatogenic series in sequential arrangement and characterized by scanty number of tails of spermatozoa in most of the lumina (LU) of seminiferous tubules, normal looking blood vessel (BV), interstitial space containing interstitial cells of Leydig (LC). The average luminal-tubular ratio is 1:2.

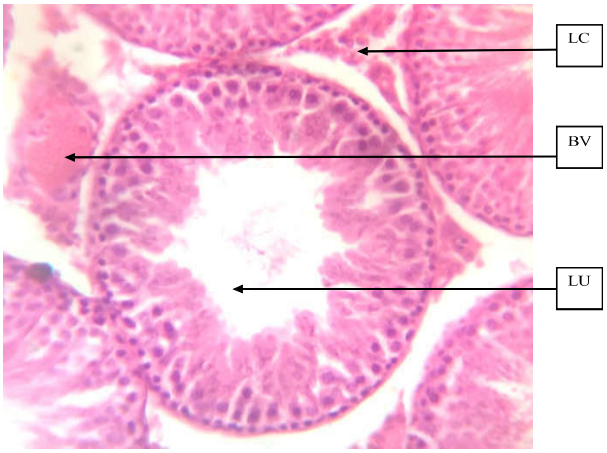

Fig. 4: Photomicrograph of cross section of testes of Group B (400mg/kg body weight/day of glyphosate) male Wistar rat (x400; H & E) showing cells of the spermatogenic series in sequential arrangement and characterized by scanty number of tails of spermatozoa in the lumen (LU) of seminiferous tubule, normal looking blood vessel (BV), interstitial space containing interstitial cells of Leydig (LC). The series appeared separated apart when compared with control group. The luminal-tubular ratio is 1:2.

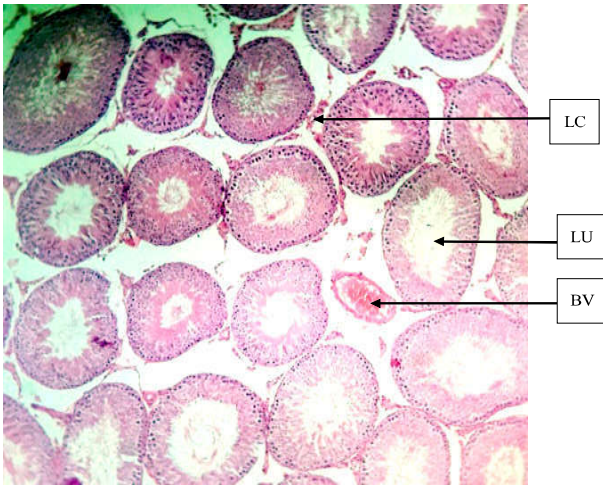

Fig. 5: Photomicrograph of cross section of testes of Group C (2000mg/kg body weight/day of glyphosate) male Wistar rat (x100; H & E) showing cells of the spermatogenic series in sequential arrangement and characterized by scanty number of tails of spermatozoa in most of the lumina (LU) of seminiferous tubules, normal looking blood vessel (BV), widening of interstitial space with dispersed interstitial cells of Leydig (LC) compared to that of control. The average luminal-tubular ratio is 1:2.

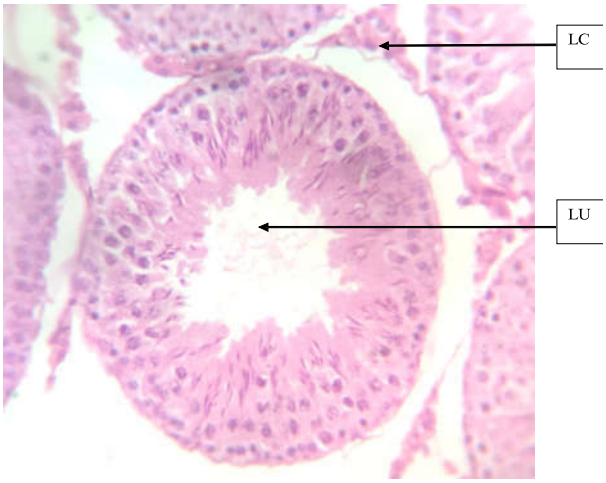

Fig. 6: Photomicrograph of cross section of testes of Group C (2000mg/kg body weight/day of glyphosate) male Wistar rat (x400; H & E) showing cells of the spermatogenic series in sequential arrangement and characterized by scanty number of tails of spermatozoa in the lumen (LU) of seminiferous tubule, interstitial space containing interstitial cells of Leydig (LC). The series are widely separated compared to that of control. The luminal-tubular ratio is 1:2.

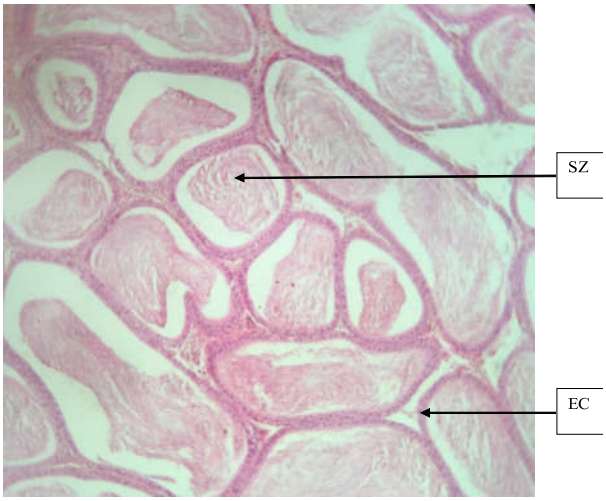

Fig. 7: Photomicrograph of cross section of caudal epididymis of Group A (Control) male Wistar rat (x100; H & E) showing clumps of spermatozoa (SZ) within the lumina and normal looking epithelial cells (EC). The average luminal-tubular ratio is 1:1.2.

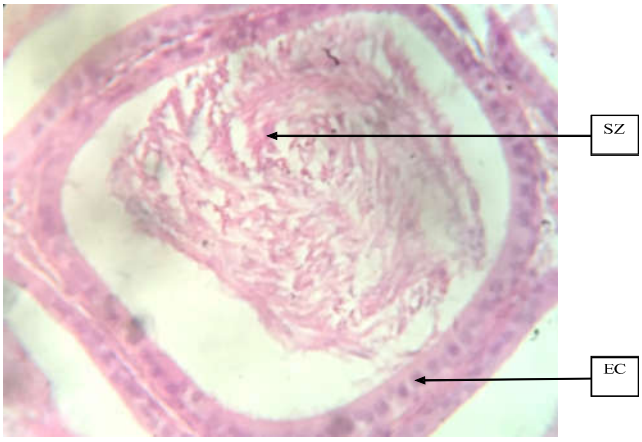

Fig. 8: Photomicrograph of cross section of epididymis of Group A (Control) male Wistar rat (x400; H & E) showing clumps of spermatozoa (SZ) within the lumen and normal looking epithelial cells (EC). The luminal-tubular ratio is 1:1.2.

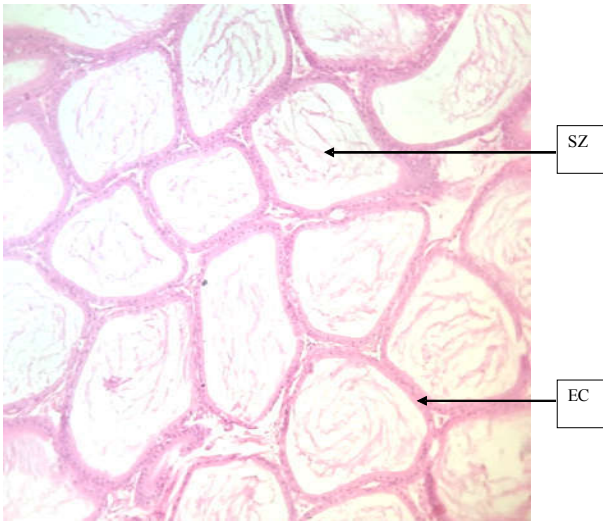

Fig. 9: Photomicrograph of cross section of epididymis of Group B (400mg/kg body weight/day of glyphosate) male Wistar rat (x100; H & E) showing scanty clumps of spermatozoa (SZ) within the lumina and normal looking epithelial cells (EC). The average luminal-tubular ratio is 1:1.1.

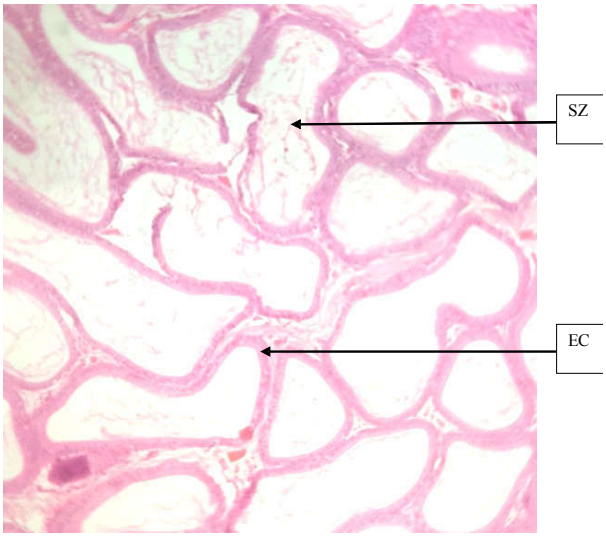

Fig. 11: Photomicrograph of cross section of epididymis of Group C (2000mg/kg body weight/day of glyphosate) male Wistar rat (x100; H & E) showing scanty clumps of spermatozoa (SZ) within the lumina and normal looking epithelial cells (EC). The average luminal-tubular ratio is 1:1.2.

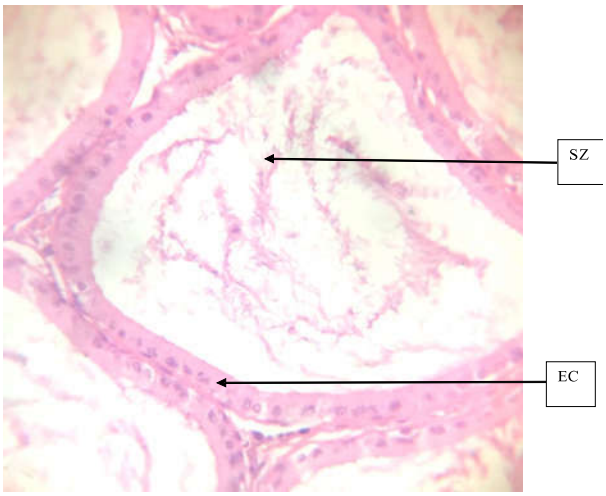

Fig. 10: Photomicrograph of cross section of epididymis of Group B (400mg/kg body weight/day of glyphosate) male Wistar rat (x400; H & E) showing scanty clump of spermatozoa (SZ) within the lumen and disruption of pseudostratified epithelial cells (EC) to cuboidal-like cell when compared with control. The luminal-tubular ratio is 1:1.2.

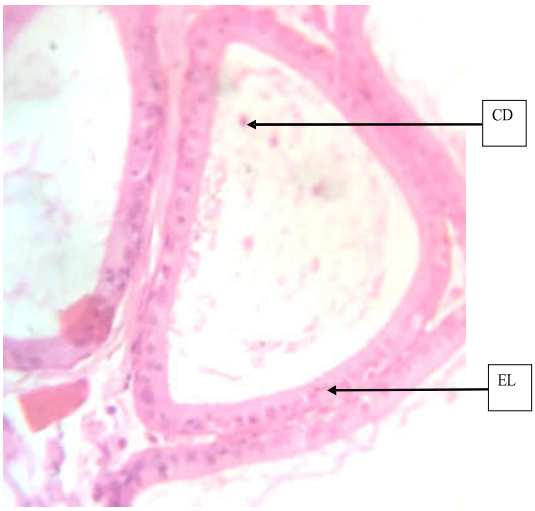

Fig. 12: Photomicrograph of cross section of epididymis of Group C (2000mg/kg body weight/day of glyphosate) male Wistar rat (x400; H & E) showing scanty cellular debris (CD), disruption of brush border and pseudostratified epithelial lining (EL) when compared with control. The average luminal-tubular ratio is 1:1.2.

Table 1: Showing results of Mammary gland staining

Rats	Mawt (g)	mSEM (g)	Mwtc (g)	Matwt (g)
A	236.89	1.872	1.8↑	1.64
B	232.42	1.084	1.0↑	1.35
C	229.76	1.185	1.1↓	1.24
p	0.001	0.031	0.035	0.007

Key
A…….Rat in control group, B…….Rat in low dose group
C…….Rat in high dose group, Mawt…..Mean average body weight (average weight in each group/5)
mSEM…Mean Standard Error of Mean (SEM in each group/5),
Mwtc.....Mean body weight change (awt in each group/5)
Matwt…..Mean testicular weight (atwt in each group/5)
↑…….Increasing in value, ↓…….Decreasing in value, g……..gram (metric unit of weight)

Discussion

The use of Wistar rats for experimental studies has been in vogue over time and results from such studies can usually be extrapolated among *Vertebrata,* including *Homo sapiens loquens.* The gradual affection of physical and behavioural changes among group B and C Wistar rats were demonstration of the toxic effects of glyphosate as previously documented [21].

Glyphosate either appeared to have penetrated the blood brain barrier (BBB) or had a prejudicious bodily physiological consequences accounting for the behavioural changes among treated rats. Excessive perspiration among rats in high-dose group C could further be attributed to the altered physiological function by stimulation of sweat gland apparatus.

The bodily weight gains in groups A and B rats could have been physiological while the decrease in group C rats might have resulted from the increasing intoxicating effect of glyphosate on the treated rats. Besides, dose-dependent decrease in mean testicular weights among group B and C rats showed that glyphosate probably exhibited direct toxic effects on testes. The higher standard error of mean (SEM) of rats in group C demonstrated a statistically-significant higher random effect of glyphosate on the body weight compared to a lesser effect on group B rats. The dose of glyphosate used in treating group B rats could then be said to be appease in terms of body weight assessment. Previous studies conducted on the effect of glyphosate in rabbits showed decline in body weight, libido, ejaculate volume, sperm concentration, semen initial fructose and semen osmolality with accompanying dead spermatozoa [22].

Some of the results with Wistar rats from the present study, for instance, the decreased weight gain among group C rats and decreased testicular weight among treated rats corroborated the previous findings with that of the rabbits.

Among the treated rats, the relative disruption of tubular epithelial linings might have contributed to the fluid accumulation or excessive secretion leading to decrease sperm density within the reproductive tract. Even though, the Leydig cells (LC) appeared normal under light microscope, the state of Sertoli cells (SC) could not be categorically ascertained since electron or other highly-resolute microscopes for the cellular ultra-structure needed to ascertain SC and other structures were not feasible [24].

The presence of celluar debris within the testicles and epididymides of rats in treated high group C futher established the toxic effects of glyphosate in causing infertility with significant exposure as against the German's assertion that the chemical had no reproductive consequences [4]. The deduction from these histological findings showed that glyphosate probably had a direct toxic effect on sperm cells, possibly through apoptotic or necrotic cellular death mechanism [15].

Morphometric analysis of treated rats showed reduction in the average luminal-tubular ratio: increasing luminal diameters while the overall tubular diameters were decreasing. Since breadth or diameter of a tube is directly proportion to the volume and area, thereby, the estimated diameters in this index study could be likened to the volume or content within the lumen. The increasing lumina of treated groups with reduction in clumps of spermatozoa signified another matter, most especially, fluid taking the place of spermatozoa with consequent sub-fertility. The dose related decreasing density of clumps of spermatozoa then showed that the higher the dose of glyphosate the lesser the spermatozoa in the reproductive tract. The relative cytoskleletal architectural disruption of the tubular epithelial layers among rats in treated groups suggested that glyphosate either had direct effect on blood-testis-barrier (BTB) or direct toxic effect on the spermatogenic series and the luminal volumetrics.

Conclusion/Recommendation

The findings from this research work suggested that glyphosate might have penetrated blood-brain barrier (BBB) of the rats leading to thier behavioural intoxication. Glyphosate had deleterious effect on body and testicular weights apart from the spermicidal histological derangement of morphometry of testis and epididymis with consequential reduction of spermatozoa density within the reproductive tract. Put together, glyphosate might have crossed the blood-testis-barrier (BTB) to affect epithelial linings of the male reproductive tracts in treated rats, thereby, corroborating the anti-fertility effect of glyphosate.

The various issues surrounding the banning of glyphosate in some countries should be finalized by intensifying research on the effects of glyphosate on other organ-systems in order to formulate a policy in this part of the world on whether glyphosate should be banned or discouraged. Besides, further studies on the histology using electron microscopic studies of the testis and epididymis should be carried out.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were revealed.

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References

1. Aktar W, Sengupta D, Chowdhury A. Impact of pesticides use in agriculture: their benefits and hazards. Interdisciplinary Toxicology 2009;2(1):1-12.
2. Rowe P J, Comhaire F H, Hargreave T B, Mahmoud A MA. "Chapter 2: History taking". WHO manual for the standardized investigation, diagnosis and management of the infertile male. Cambridge [England]: Published on behalf of the World Health Organization by Cambridge University Press 2000. pp.5–16.
3. Anadón A, Martínez-Larrañaga MR, Martínez MA, Castellano VJ, Martínez M, Martin MT, Nozal M J, Bernal JL. Toxicokinetics of glyphosate and its metabolite aminomethyl phosphonic acid in rats. Toxicology Letters 2009;190(1):91-5.
4. Glyphosate: Efsa Updates Toxicological Profile. European Food Safety Authority. https://www.efsa.europa.eu/en/press/news/151112. Accessed: 30/08/2016.
5. Mae-Wan H. Glyphosate/Roundup and Human Male Infertility. Health and Disease. http://www.isis.org.uk/full/Glyphosate_Roundup_and_ Human_Male_InfertilityFull.php. Accessed: 12/01/ 2016.
6. Plant T M. The hypothalamo-pituitary-gonadal axis. Journal of Endocrinology 2015;226:41-54.
7. Jungwirth A, Diemer T, Dohle G R, Giwercman A, Kopa Z, Krausz C, Tournaye H. European Association of Urology Guidelines on Male Infertility. http://uroweb.org/wp-content/uploads/17-Male-Infertility_LR1.pdf. Accessed: 01/09/2016.
8. Gudeloglu A, Parekattil S J. Update in the evaluation of the azoospermic male. Clinics (Sao Paulo) 2013; 68(1):27-34.
9. Centers for Disease Control and Prevention. Guidance on microbial contamination in previously flooded outdoor areas. Atlanta: U.S. Department of Health and Human Services. https://www.cdc.gov/nceh/ehs/publications/guidance_flooding.htm. Accessed: 05/07/2016.
10. Shaner D L, Nadler-Hussar T, Henry W B, Koger C H. A rapid in vivo shikimate accumulation assay with excised leaf discs. Weed Science 2005;53(6):769-74.
11. Shaner D. An overview of glyphosate mode of action: why is it such a great herbicide? North Central Weed Science Society Proceedings 2006;61:94.
12. Cavalli VLLO, Cattani D, Rieg CEH, Pierozan P, Zanatta L, Parisotto E B, Filho DW, Silva FRMB, Pessoa-Pureur R And Zamoner A. Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. Free Radical Biology and Medicine 2013;65:335-46.
13. Ermak G, Davies K J A. Calcium and oxidative stress: from cell signalling to cell death. Molecular Immunology 2002;38:713-21.
14. Lemasters J L, Nieminen A, Qian T, Trost L C, Elmore S P, Nishimura Y et al. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochimica et Biophysica Acta (BBA) – Bioenergetics 1998;366(12): 177-196.
15. Zhivotovsky B, Orrenius S. Calcium and cell death mechanism: A perspective from the cell death community. Cell Calcium 2011;50:211-21.
16. Schuette J. Environmental fate of glyphosate. Environmental monitoring and pest management 1998.pp.1-13.
17. United States Environmental Protection Agency. Office of Pesticide Programs. Special Review and Reregistration Division. Reregistration eligibility decision (RED): Glyphosate. Washington District of Columbia (September). http://web.net/nwatch/fmp/workshops/herbicideworkshop/Glyphosate. pdf. *Accessed: 30/08/2016.*
18. Cox C. Glyphosate, Part 1: Toxicology. Journal of Pesticide Reform 1995;15(3): -27.
19. Ikebuaso A D, Yama O E, Duru F I O, Oyebadejo S A. Experimental Testicular Torsion in a Rat Model: Effects of Treatment with Pausinystalia macroceras on Testis Functions. Journal of Reproduction and Infertility 2012;13(4):218-24.
20. Hess R A, Moore B J. Histological Methods for the Evaluation of the Testis**.** In book: Methods in Reproductive Toxicology. Publisher: Academic Press, Editors: Chapin, R.E, Heindel, J.J, 1993.pp.52-85.
21. Webster T U, Laing L V, Florence H, Santos E M. Effects of Glyphosate and its Formulation, Roundup, on Reproduction in Zebrafish (Danio rerio). Environmental Science Technology 2014;48:1271-79.
22. Yousef M I, Salem M H, Ibrahim H Z, Helmi S, Seehy M A, Bertheussen K. Toxic effects of Carbofuran and Glyphosate on semen characteristics in rabbits. Journal of Environmental Sciences and Health Biostatics 1995;30(4):513-34.
23. Young B, Lowe J S, Stevens A. Male reproductive system. In: Wheater's Functional Histology. 5ed Elsevier 2007.pp.346-58.