Original Research

Testicular Toxicity Induced by Glyphosate (GLP) and Ameliorative Effect of Vitamin C in Wistar Rats

M. Lakshmi Namratha[1], M. Lakshman[1*], M. Jeevanalatha[2] and B. Anil Kumar[3]

[1]Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad-500030, INDIA
[2]Department of Veterinary Pathology, College of Veterinary Science, Mamnoor-506166, INDIA
[3]Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Rajendranagar, Hyderabad-500030, INDIA

***Corresponding author:** mekala_bry@yahoo.com

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Abstract

The aim of the present experiment was to study the testicular toxicity induced by glyphosate and the protective role of Vitamin C in male albino Wistar rats. Forty eight (48) rats were randomized into four groups consisting of twelve (12) animals in each: Group 1 served as control, group 2 received glyphosate at a dose of 500 mg/kg b.wt, group 3 received Vitamin C at a dose of 250 mg/kg b.wt and group 4 received glyphosate (500 mg/kg b.wt) and Vitamin C (250 mg/kg b.wt). The treatment regimens were administered by oral gavage once daily for three weeks. The animals were sacrificed on 7th and 21st day post treatment. Results showed a significant increase in the percentage of abnormal sperms, reduction in the levels of testosterone and histopathological changes in the testes of glyphosate treated rats. However, administration of Vitamin C caused a mild to moderate ameliorative effect on the parameters investigated.

Key words: Glyphosate, Testicular Toxicity, Testosterone and Histopathology, Vitamin C

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Introduction

Glyphosate is the active ingredient of Roundup®, marketed as a non-selective, broad spectrum, post emergence herbicide. It is used to control the weeds in emerged grasses, rice, corn and soy plantations (Smith and Oehme, 1992). Previous studies (Ikpeme *et al.*, 2012 and Richard *et al.*, 2005) showed that the GLP has serious toxic effects on health and environment. The GLP being an endocrine disruptor in human cells; it has induced DNA damage and interferes with physiological DNA repair machinery, leading to genomic instability and cancer development. The GLP has the ability to suppress the activities of Cytochrome P-450 family, including Cyp-450 aromatase enzyme which indicates a possible inhibitory

effect on synthesis of sex steroids by the Leydig cells in testes. Toxic effects of GLP could be due to uncoupling of oxidative phosphorylation.

Vitamin C is involved in the prevention of cellular damage by safely interacting with the free radicals and terminating the chain reactions before vital molecules are damaged (Ikpeme *et al.*, 2012). Hence, the present study was designed to study the GLP induced testicular toxicity and its amelioration with Ascorbic Acid (AA) in male albino *Wistar* rats.

Materials and Methods

Experimental Animals

Forty-eight adult male albino *Wistar* rats weighing 200-240 g, bred at Jeeva Life Sciences (ISO 9001:2015 certified company), Hyderabad were used for this research. The rats were housed in solid bottom polypropylene cages at RUSKA Labs, Department of Veterinary Pathology and were maintained in controlled environment (20-22^0 C) throughout the course of experiment. Sterile rice husk was used as standard bedding material. All the rats were provided with standard pellet diet (low fat and nutritionally balanced food) and deionized drinking water *ad libitum* throughout the experimental period. The experiment was carried out according to the guidelines and prior approval of Institutional Animal Ethics Committee (IAEC-No.01-2019).

Chemical Source

Glyphosate was obtained from Seed Research and Technology Centre (SRTC), Professor Jayashankar Telangana State Agriculture University (PJTSAU), Hyderabad-30 under the trade name Roundup® (41%) and Vitamin C was obtained from S.D. Fine-Chem Ltd., Mumbai, India.

Experimental Design

A total of 48 male albino *Wistar* rats were randomly divided into four (4) groups consisting of twelve (12) animals in each.

Group 1 - Control

Group 2 - GLP (@500 mg/kg b.wt)

Group 3 - AA (@250 mg/kg b.wt)

Group 4 - GLP+AA (@500 mg/kg b.wt + 250mg/kg b.wt)

The dose regimens were administered *per os* once daily for a period of three weeks. The rats were monitored for clinical signs and death. The experiment was carried out for 21 days and six animals from each group were sacrificed on 7th and 21st day of experiment.

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Sperm Morphology Assay

The cauda epididymis of one testis was excised and placed in a sterile petri dish containing 2 mL warm (37^0C) normal saline then it was macerated by sterile scissors to obtain the epididymis contents in a suspension and used for semen analysis (Hafez, 1970). One drop of undiluted and liquefied semen was mixed in a ceramic well with one drop of the Eosin Nigrosin staining solution (50 µL). The suspension was incubated for 30 seconds at room temperature (20-25^0C). Then, 12 µL suspension was transferred with micro-pipette onto a labelled microscope slide for preparation of thick smear. Two smears were prepared from each sample, air dried and examined directly under Light Microscope (LM) by using 100x (Oil immersion) and counted around 200-250 sperms.

Hormonal Assay

Serum samples collected from blood were preserved at -20^0C and was analyzed for testosterone by using ELISA kit.

Histopathology

The tissue samples of testes (1×1 cm^3) were collected and fixed in 10% Neutral Buffered Formalin (NBF) soon after necropsy. The samples were processed, sectioned (5 µm) and stained with Hematoxylin and Eosin (H&E) for histopathological examination as per the standard procedure (Luna, 1968).

Statistical Analysis

Data obtained were subjected to statistical analysis by applying one way ANOVA using statistical package for social sciences (SPSS) version 16.0. Differences between the means were tested by using Duncan's multiple comparison tests and significance level was set at $P<0.05$ (Snedecor and Cochran, 1994).

Results and Discussion

Effect of GLP on Sperm Morphology

A significant ($P<0.05$) increase in the mean values of abnormal sperms were recorded on 7^{th} day post treatment in groups 2 and 4 (39.83 ± 2.62 and 33.5 ± 1.64) as compared to groups 1 and 3 (15.17 ± 1.13 and 13.17 ± 1.01). Similarly, on 21^{st} day post treatment there was a significant ($P<0.05$) increase in abnormal sperms in groups 2 and 4 (56.17 ± 2.27 and 44.67 ± 1.38) when compared to the groups 1 and 3 (17.83 ± 1.44 and 13.67 ± 1.56), a significant difference was also noticed between the groups 2 and 4 on 7^{th} and 21^{st} day of experiment (Table 1). The groups 1 and 3 rats testes showed normal sperms with hook shaped head with a long tail and intact mitochondrial sheath (Fig. 1). The abnormal sperm cells were noticed in groups 2 and 4 on 7^{th} and 21^{st} day of experiment which include bent heads, midpiece and tails (Fig. 2, 7 and 8), detatched heads (Fig. 3 and 10), amorphous heads (Fig. 4), banana shaped heads (Fig. 6), broken

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necks (Fig. 5), decapitated sperms (Fig. 10), tail coiling (Fig. 8, 9 and 12), presence of cytoplasmic droplets (Fig. 12) and absence of mitochondrial sheath (Fig. 11).

Table 1: Sperm morphology (%) in different groups

Group	Day 7	Day 21
Group 1	15.17 ± 1.13[a]	17.83 ± 1.44[a]
Group 2	39.83 ± 2.62[c]	56.17 ± 2.27[c]
Group 3	13.17 ± 1.01[a]	13.67 ± 1.56[a]
Group 4	33.5 ± 1.64[b]	44.67 ± 1.38[b]
P value	*	*

Values are Mean ± SE (n=6); One-way ANOVA; Means with different superscripts in a column differ significantly at P<0.05 (*)

Head and Tail Abnormalities

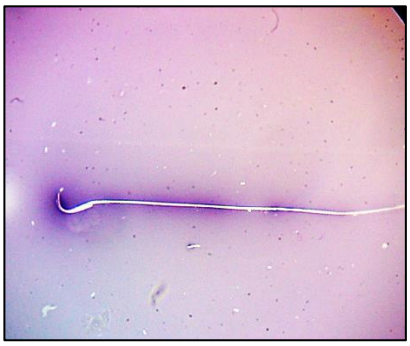

Fig.1: Normal spermatozoa showing hook shaped head and intact mitochondrial sheath (Group 1, Day 21): Eosin Nigrosin x1000

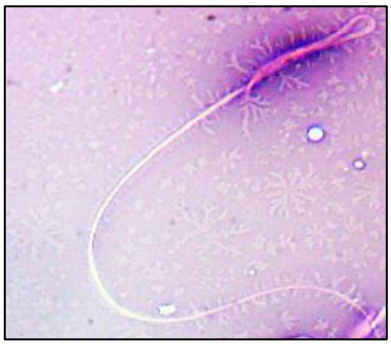

Fig.2: Bent neck and head (Group 2, Day 7): Eosin Nigrosin x1000

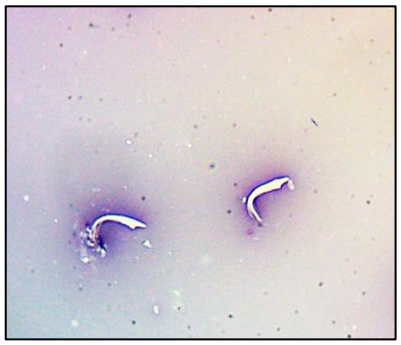

Fig.3: Detatched heads (Group 2, Day 7): Eosin Nigrosin x1000

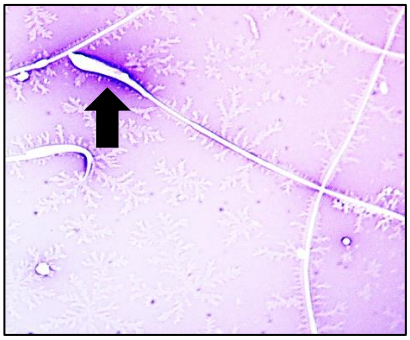

Fig.4: Amorphous head (arrow) (Group 2, Day 21): Eosin Nigrosin x1000

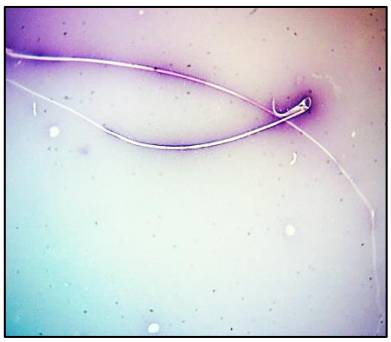

Fig.5: Broken neck (Group 2, Day 21): Eosin Nigrosin x1000

Fig.6: Banana shaped sperm head (Group 2, Day 21): Eosin Nigrosin x1000

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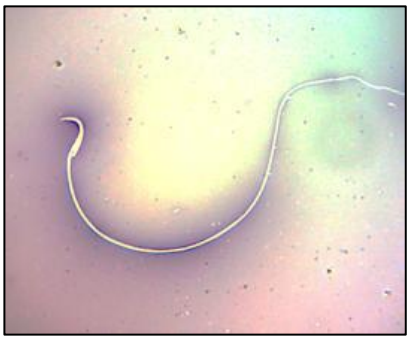

Fig.7: Bent midpiece (Group 2, Day 21): Eosin Nigrosin x1000

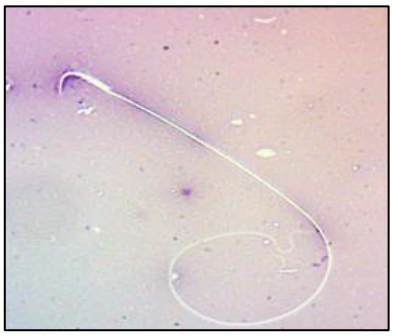

Fig.8: Bent tail with mild terminal coiling (Group 4, Day 7): Eosin Nigrosin x1000

Fig.9: Tail coiling (Group 4, Day 21): Eosin Nigrosin x1000

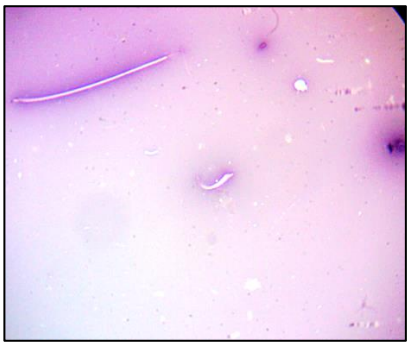

Fig.10: Decapitated sperm and detached head (Group 4, Day 21): Eosin Nigrosin x1000

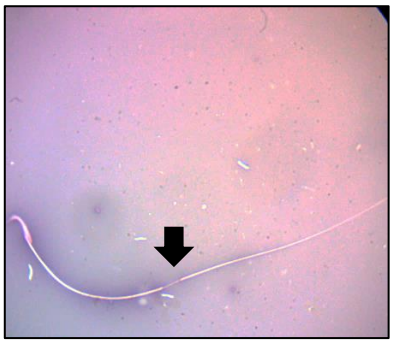

Fig.11: Absence of mitochondrial sheath (arrow) (Group 4, Day 21): Eosin Nigrosin x1000

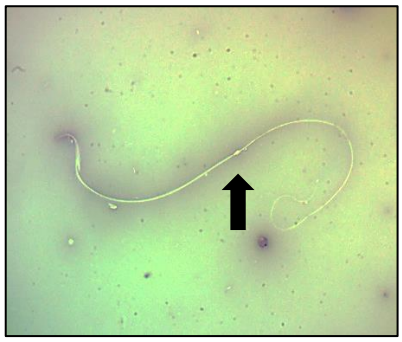

Fig.12: Bent tail with terminal coiling and presence of distal cytoplasmic droplet (arrow) (Group 4, Day 21): Eosin Nigrosin x1000

In the present experiment, a significant ($P<0.05$) increase in the abnormal sperm count was noticed in glyphosate treated rats as compared to control rats throughout the experimental period. The results are in accordance with the findings of Dallegrave *et al.* (2007), Ikpeme *et al*. (2012), Owagboriaye *et al.* (2017) and Avdatek *et al.* (2018). The occurrence of sperm head abnormalities has been attributed to the chromosomal aberrations that occur during the packaging of genetic material in the sperm head or occurrence of a point mutation in testicular DNA (Bruce and Heddle, 1979). Thus, glyphosate interacts with the genetic material in the testes of the exposed rats during spermatogenesis which probably leads to increased number of abnormal sperm cells (Owagboriaye *et al.*, 2017). The significant changes in the bio-molecules are responsible for irreparable damage to major spermatogenic cells and Leydig cells. It also affected the androgen biosynthesis pathways. Thereby leading to decreased testosterone levels which might have interfered with spermatopoiesis, resulted in increased number of abnormal sperms and subsequently affecting the male fertility.

Less number of abnormal sperms were noticed in ameliorative group (Group 4) in which less intoxicated germinal cells (Reparable damaged cells) compared with group 2 on 7th and 21st day of experiment. The

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Vit. C might have interfered in reconstruction of less damaged germinal cells. These findings are in line with the observations of Ikpeme *et al*. (2012).

Effect of GLP on Hormonal Assay

The mean values of testosterone in different groups (1, 2, 3 and 4) were ranged from 1.53 ± 0.06, 1.09 ± 0.04, 1.57 ± 0.03 and 1.27 ± 0.06 on day 7th and 1.59 ± 0.02, 0.91 ± 0.02, 1.6 ± 0.02 and 1.31 ± 0.08 on 21st day of experiment. Significantly (P<0.05) decreased testosterone levels were recorded in groups 2 and 4 on 7th and 21st day as compared to group 1 and group 3. A significant difference was also noticed between the groups 2 and 4 on 7th and 21st day of experiment (Table 2).

Table 2: Testosterone (ng/mL) in different groups

Group	Day 7	Day 21
Group 1	1.53 ± 0.06^{c}	1.59 ± 0.02^{c}
Group 2	1.09 ± 0.04^{a}	0.91 ± 0.02^{a}
Group 3	1.57 ± 0.03^{c}	1.6 ± 0.02^{c}
Group 4	1.27 ± 0.06^{b}	1.31 ± 0.08^{b}
P value	*	*

Values are Mean ± SE (n=6); One-way ANOVA; Means with different superscripts in a column differ significantly at P<0.05 (*)

In the present study, a significant (P<0.05) reduction in testosterone levels were recorded in group 2 over group 1, which are in accordance with the observations of previous authors (Romano *et al.*, 2010; Dai *et al.*, 2016; Youness *et al.*, 2016 and Owagboriaye *et al.*, 2017). Mature rats testicular cells exposed to glyphosate and Roundup® at lower levels showed a decrease in the concentration of testosterone by 35 per cent *in vitro* (Clair *et al.*, 2012). In drakes and cell cultures treated with Roundup® also had a decrease in the levels of testosterone (Oliveira *et al.*, 2007 and Walsh *et al.*, 2000). The reduction in steroid hormones could be due to Roundup® which interfered with the different steps of the biosynthesis of sex steroids (Walsh *et al.*, 2000). These effects include reduction in Steroidogenic Acute Regulatory (StAR) protein expression (Walsh *et al.*, 2000) and reduction in aromatase mRNA and its activity, culminating in decrease of estradiol synthesis, as seen in *in vitro* experiments (Richard *et al.*, 2005). Therefore, the reduction in testosterone levels observed in the present study suggests that the GLP might have acted as an endocrine disruptor leading to reduced levels of testosterone thereby affecting male fertility.

A mild to moderate improvement was noticed in ameliorative group (Group 4) as compared to glyphosate treated group (Group 2) indicating the protective role of Vit. C against GLP induced toxicity to some extent.

Effect of GLP on Histopathology

Light microscopic examination of testes sections from group 1 showed rounded seminiferous tubules bounded by a fibrous capsule. The seminiferous tubules were lined with spermatogenic layer. Sertoli cells

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are triangular in shape with vesicular nuclei. The lumen of the tubules were filled with spermatozoa. The space between the tubules contained connective tissue and Leydig cells (Fig.1). Different sections of testes from group 2 on 7th day showed mild interstitial edema, shrunken Leydig cells with pyknotic nuclei, distorted and thin basement membrane, varied sizes of Sertoli cells, degenerated spermatids and spermatocytes (Fig. 2 and 3). On 21st day, hemorrhage, congestion, MNC infiltration degeneration of germinal epithelium, karyorrhectic nuclei and presence of eosinophilic mass in the lumen (Fig. 4 and 5). The sections of testes from group 3 showed an increased number of spermatogonia and spermatocytes. The interstitial Leydig cells were evidenced (Fig.6).

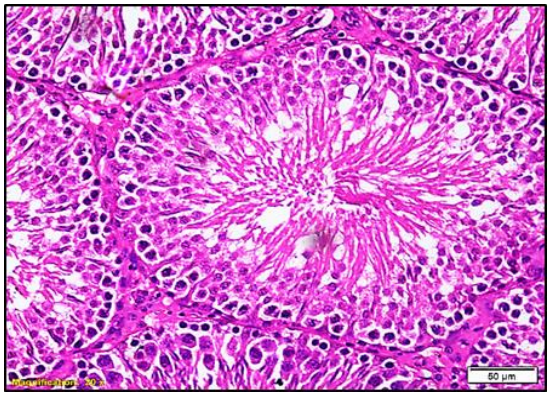

Fig.1: Photomicrograph of testis showing normal feature of seminiferous tubules with active spermatogenesis and interstitial space with Leydig cells (Group 1, Day 7): H&E x200

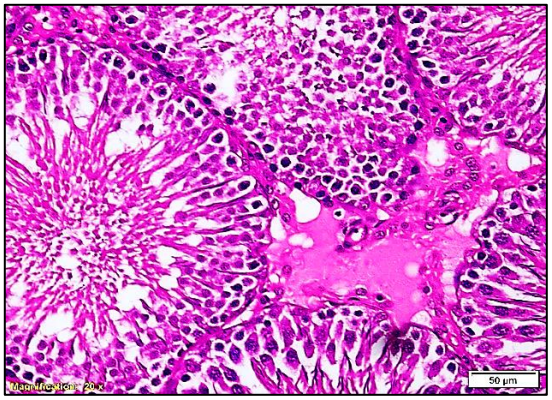

Fig.2: Photomicrograph of testis showing mild interstitial edema, shrunken Leydig cells with pyknotic nuclei, distorted and thin basement membrane, varied sizes of Sertoli cells, degenerated spermatids and spermatocytes (Group 2, Day 7): H&E x200

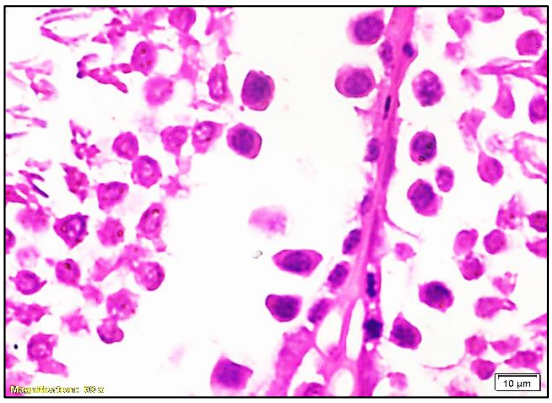

Fig.3: Photomicrograph of testis showing Sertoli cell degeneration, distortion of spermatocytes, spermatids and thin basement membrane (Group 2, Day 7): H&E x600

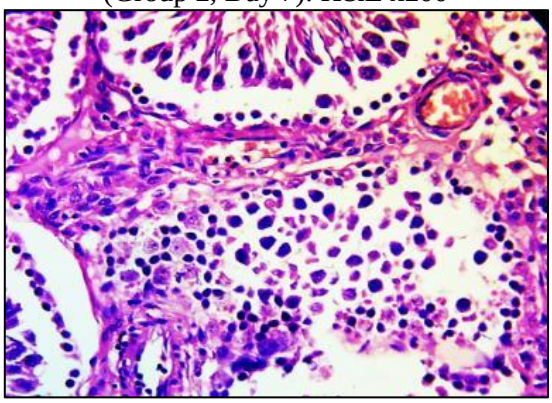

Fig.4: Photomicrograph of testis showing hemorrhage, congestion and MNC infiltration (Group 2, Day 21): H&E x400

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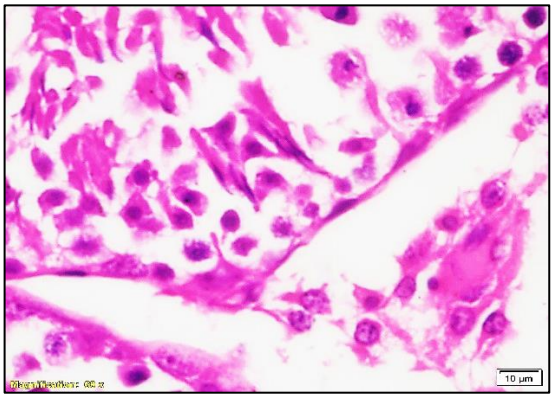

Fig.5: Photomicrograph of testis showing degeneration of germinal epithelium, karyorrhectic nuclei and presence of eosinophilic mass in the lumen (Group 2, Day 21): H&E x600

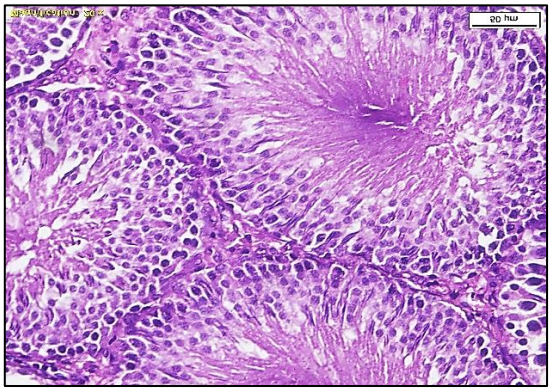

Fig.6: Photomicrograph of testis showing normal feature of seminiferous tubules with active spermatogenesis and interstitial space with Leydig cells (Group 3, Day 21): H&E x200

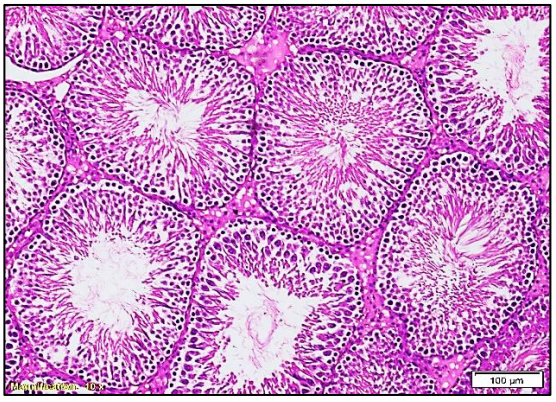

Fig.7: Photomicrograph of testis showing mild intertubular edema with regeneration of Sertoli cells and active spermatogenesis (Group 4, Day 7): H&E x100

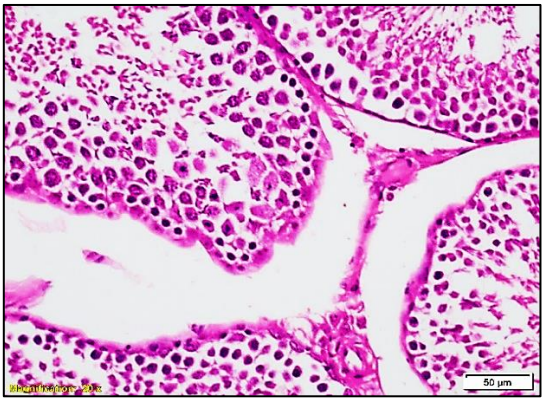

Fig.8: Photomicrograph of testis showing Leydig cell degeneration, necrosis of germinal epithelium and degenerated spermatids (Group 4, Day 21): H&E x200

The testicular sections of group 4 showed less defects than treated group (Group 2). There was regeneration of germinal epithelium, Sertoli cells and active spermatogenesis on 7th day (Fig.7). On 21st day, sections revealed congestion, necrosis of Leydig cells, degeneration, necrosis of germinal epithelium and decreased number of spermatids (Fig.8).

Degenerative changes in the seminiferous tubules and interstitial Leydig cells are evidence of Roundup induced toxicity to the male reproductive system of rats. These findings are in accordance with the earlier observations (Ikpeme *et al.*, 2012; Youness *et al.*, 2016; Owagboriaye *et al*., 2017 and Avdatek *et al.*, 2018). Hypothetically, these degenerative changes in the seminiferous tubules might have hampered the spermatogenesis process leading to decreased concentration of sperms and also the production of abnormal sperms thereby affecting male fertility. There were minimal lesions in the testes sections of group 4 rats administered with Vitamin C along with GLP when compared to group 2 which shows that AA might have mopped up the released free radicals, thus maintaining the integrity of the cells to some extent.

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Conclusion

Overall the present study suggests that the GLP intoxication can cause severe damage to testes leading to increased number of abnormal sperms, reduced levels of testosterone and histopathological changes in testes are also evidenced. Administration of Vitamin C alone has not completely ameliorated the toxic changes induced by GLP despite of its high antioxidant properties.

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