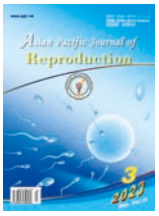

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Effects of a glyphosate-based herbicide on the oestrous cycle of rats

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ABSTRACT

Objective: To determine the effects of a glyphosate-based herbicide on the oestrous cycle of rats.

Methods: Fifteen adult Wistar rats were randomly divided into three groups. Group A served as the control group, while groups B and C received 250 and 500 mg/kg of glyphosate-based herbicide orally for five oestrous cycles, respectively. Stages of oestrous cycle, oestrous cycle index, length of cycle, oestrous cycle ratio, serum estradiol and progesterone levels were determined.

Results: The proestrus and oestrus stages of the glyphosate-based herbicide groups increased significantly (P<0.05), although proestrus in group C was not significantly different from the control group. There was a significant decrease in the metestrus and diestrus of the glyphosate-based herbicide groups (P<0.05). The oestrous cycle index of the glyphosate-based herbicide treated groups was altered; this was characterized by an increase in the oestrous index and a decrease in the metestrus and diestrus indexes. The proestrus index of group B increased, while that of group C decreased. The length of the cycle of the glyphosate-based herbicide groups significantly decreased from the 1st and 3rd week till the end of the study in groups B and C, respectively (P<0.05). There was a significant increase in the oestrous cycle ratio of the glyphosate-based herbicide groups compared to the control group (P<0.05). Though the estradiol and progesterone levels of the glyphosate-based herbicide groups increased and decreased, respectively, they were not significantly different from the control group.

Conclusions: Glyphosate-based herbicide at the doses of 250 and 500 mg/kg can alter the pattern of the oestrous cycle in rats.

KEYWORDS: Estrogen; Glyphosate-based herbicide; Oestrous cycle; Oestrus; Progesterone

1. Introduction

Herbicides are chemical compounds used commercially by farmers due to their ability to kill or destroy plants, especially weeds[1]. Glyphosate is the most widely used herbicide in agriculture[2]. It is a broad-spectrum herbicide effective against weeds, especially in association with transgenic glyphosate-resistant crop systems[3]. Glyphosate has both acidic (pH<2.0, 2.6, 5.6) and alkaline (pH 10.6) properties[4] (the World Health Organization, 1994), and is often mischaracterized as an organophosphate, due to its molecular structure which has an organic molecule containing a phosphorus atom. However, clinical signs of toxicity in man and animals do not reflect the classical symptoms of organophosphate poisoning[5].

Glyphosate acts by inhibiting enolpyruvylshikimate phosphate synthase, an enzyme required to synthesize several essential aromatic amino acids in the shikimate pathway[6]. This metabolic pathway is common to all plants, making glyphosate an effective, non-selective herbicide. However, it is supplemented with adjuvants, which are together referred to as glyphosate-based herbicides and are aromatase disruptors in different tissues and species of animals[5,7].

Significance

Glyphosate-based herbicides are broad-spectrum herbicides used against weeds. Previous studies suggest glyphosate-based herbicides have adverse effects on the metabolic process of man and animals. This study showed that glyphosate-based herbicide alters the oestrous cycle in rats characterized by changes in the oestrus, oestrous cycle indices, length of the oestrous and oestrous cycle ratio. This finding implies that the reproductive cycle of females exposed to glyphosate-based herbicides at the doses of 250 and 500 mg/kg may also be altered.

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The indiscriminate application of herbicides by illiterate farmers poses a threat to both human and animal health manifesting as cancer and neurodegenerative, reproductive and developmental changes and respiratory effects[8,9]. The residual effect slowly accumulates in the body disrupting metabolic activities[9]. This poses a significant threat to the environment affecting crop yield, man and animal health. Many doses of glyphosate have residual effects which can slowly accumulate in the system of an animal, disrupting metabolic activities within the body[10]. Glyphosate when released into circulation can mimic estrogen to increase animal infertility by alteration of the oestrous cycle, leading to premature birth and birth defects[11]. This study was therefore designed to determine the effects of glyphosate on the oestrous cycle of female Wistar rats.

2. Materials and methods

2.1. Study location

The study was carried out from January to April 2020 at the Theriogenology Laboratory, Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto, Nigeria. Sokoto is in the Sahel region of Nigeria and lies between 4 °E to 6 °E and between 11 °N to 13 °N. The temperature ranges from about 14 ℃–45 ℃, with an annual average of 28.3 ℃ and relative humidity of 48%[12].

2.2. Study animals

Fifteen female Wistar rats about 3 months old weighing between 100 to 120 g were purchased from Zaria, Nigeria and transported in plastic cages by road to Sokoto (about 236 miles), during which they were fed and water provided. The rats were kept in cages and acclimatized for 14 days. Pelleted feeds (Vital feed® growers mash) and clean water *ad libitum* were provided throughout the study.

2.3. Source of glyphosate

The glyphosate used is FORCE UP®, manufactured by Zhejiang Xinan Chemical Industrial Group Co., Ltd Company. It was purchased from a distributor at the Sokoto central market. Based on the manufacturer's insert, the FORCE UP® contains 360 g of glyphosate/L in the form of 480 g/L glyphosate isopropylamine salt.

2.4. Determination of median lethal dose (LD_{50})

LD_{50} was determined through a two-phase approach as described by Lorke[13]. The LD_{50} was considered to be greater than 5 000 mg/kg because all the rats were alive 24 h after administration; therefore, the doses used were 5% and 10% of 5 000 mg/kg, which was equal to 250 mg/kg and 500 mg/kg glyphosate-based herbicide, respectively.

2.5. Study design

The fifteen female Wistar rats were monitored by vaginal cytology as described by Cooper *et al*[14] and by the appearance of the vagina according to the methods of Champlin *et al*[15] for two complete oestrous cycles, after which they were randomly divided into three groups comprising five rats each. Group A served as the control group, while groups B and C were orally administered 250 mg/kg and 500 mg/kg of glyphosate-based herbicide daily between 8:00-10:00 am for five complete oestrous cycles (20 days). During the exposure period, vaginal cytology was used daily to stage the oestrous. The number of occurrences of each stage of the oestrous cycle was recorded, and the index for each stage was calculated. The length of the oestrous cycle was determined. At the end of the study, blood was collected by cardiac puncture for hormonal analysis.

2.6. Determination of oestrous cycle

Following physical restraint, a vaginal specimen was obtained by inserting a moist cotton bud into the vaginal opening. The swab was gently rotated within the vagina of the rat, ensuring it maintain contact with the vaginal wall. The swab was removed from the vagina and rubbed on a clean glass slide to transfer the vaginal specimen to the slide. It was allowed to air dry for 3-5 min, after which 0.1% aqueous solution of methylene blue was used to stain the vaginal smear. The stained smear was allowed to dry for 3-5 min before viewing under the microscope at ×10 magnification. The cell types were used to determine the stage of the oestrous as follows: 1) Proestrus: the presence of small round nucleated epithelial cells that are deeply basophilic either in clusters (grape clusters), sheath or strands. 2) Oestrus: the presence of anucleated keratinized epithelial cells and occasionally a few nucleated epithelial cells. 3) Metestrus: a combination of anucleated keratinized epithelial cells and neutrophils with small-sized multilobulated nuclei. 4) Diestrus: few anucleated keratinized epithelial cells combined with neutrophils which are small and large nucleated epithelial cells and mucous.

For more certainty of the stage of the oestrous cycle, the appearance of the vagina was observed before taking the smear according to the methods of Champlin *et al*[15]. This method permits observations without mechanical manipulation of the vaginal tissue. 1) Proestrus: the vagina is gapping, and the tissues appear reddish-pink and moist. Numerous longitudinal folds or striations are visible on the lips. 2) Estrus: similar to proestrus, but the tissues are lighter pink and less moist, and the striations are more pronounced. 3) Metestrus: vaginal tissues are pale and dry. 4) Diestrus: vagina has a small opening, and the tissues are bluish-purple in colour and very moist.

2.7. Oestrous cycle index

The index of each stage of the oestrous cycle was calculated as: 1) Proestrus index=the number of days with proestrus smear divided

by the total duration of treatment and multiplied by 100. Estrus index=the number of days with oestrus smear divided by the total duration of treatment and multiplied by 100. 3) Metestrus index=the number of days with metestrus smear divided by the total duration of treatment and multiplied by 100. 4) Diestrus index=the number of days with diestrus smear divided by the total duration of treatment and multiplied by 100[16].

2.8. Length of oestrous cycle

It is the period between two successive heats (oestrus).

2.9. Determination of oestrous cycle ratio

The oestrous cycle ratio was calculated by dividing the summation of the number of proestrus and oestrus days by the sum of the days the rats were in metestrus and diestrus, as previously described[17].

2.10. Hormone analyses

The blood was centrifuged at 808.5×*g* for 5 min, serum was harvested and stored at -20℃ until use. The serum was used to assay for estrogen and progesterone using the Accu Bind® ELISA kit manufactured by Monobind Inc. and by following the manufacturer's instructions. The serum and all reagents were brought to room temperature before processing the assay.

2.10.1. Estrogen analysis

Twenty-five microlitres of serum reference and specimen were pipetted into the coated microplate, and 50 µL of estradiol biotin was added and swirled for 20-30 s to mix. This was then covered and incubated for 30 min at room temperature. After incubation, 50 µL of estradiol enzyme reagent was added, swirled for 20-30 s and covered to incubate for 90 min at room temperature. At the end of this period, the contents of the microplate were decanted and 350 µL of wash buffer was used for washing twice, decanting in each case. After this, 100 µL of substrate solution was added and incubated for 20 min at room temperature. The reaction was stopped by adding 50 µL of stop solution to each well and then mixing for 15-20 s. The absorbance in each well was read within 30 min thereafter using an ELISA plate reader (model: 2100-C, made in China) at 450 nm. The results were calculated by plotting a linear graph of the absorbance of serum reference *versus* the corresponding estradiol concentration of the reference, and sample results were obtained as described by the manufacturer.

2.10.2. Progesterone analysis

Twenty-five microlitres of serum reference and the specimen were pipetted into the coated microplate, and 50 µL of progesterone enzymes reagent was added to all the wells and swirled for 10-20 s, progesterone biotin reagent 50 µL was added, swirled for 20-30 s, covered with foil paper and incubated for 60 min at room temperature. After the incubation, the content of the microplate was decanted and dried. About 350 µL of wash buffer was added twice, decanting after washing. After this, 100 µL of substrate solution was added to the mixture and incubated for 20 min at room temperature. After incubation, 50 µL of stop solution was added to each well and mixed for 15-20 s. The absorbance in each well was read within 30 min of stopping the reaction using an ELISA plate reader (model: 2100-C, made in China) at 450 nm. The result was calculated by plotting a linear graph of the absorbance of serum reference *versus* the corresponding progesterone concentration of the reference, and sample results were obtained as described by the manufacturer.

2.11. Statistical analysis

Data generated from the study were analyzed using the analysis of variance (ANOVA) on Invivostat version 3.7.0.0 (https://invivostat.co.uk). Progesterone was not normally distributed and therefore it was analysed using Kruskal-Wallis. However, the oestrous cycle index was analysed using descriptive statistics. Data in tables were expressed as mean±standard deviation (mean±SD) or number. All data of $P<0.05$ were considered statistically significant.

2.12. Ethics statement

Ethical approval for the study was obtained from the Institutional Animal Care and Use Committee (IACUC), Usmanu Danfodiyo University, Sokoto, with approval number UDUS/IACUC/2020/AUP-RO-11.

3. Results

3.1. Stage of oestrous cycle

The results of the stage of oestrous cycle based on vaginal cytology (Figure 1) are presented in Table 1. There was a significant increase in the proestrus stage of the 250 mg/kg group when compared with the control group ($P<0.05$). However, that of the 500 mg/kg group did not significantly vary from the control group ($P>0.05$). The oestrus stage significantly increased in both glyphosate-based herbicide-treated groups compared to the control group ($P<0.05$). On the other hand, the metestrus and diestrus decreased significantly in both glyphosate-based herbicide-treated groups compared to the control group ($P<0.05$).

3.2. Oestrous cycle index

The proestrus and oestrus indices of the glyphosate-based herbicide-treated groups were higher than the control group although the proestrus of the 500 mg/kg group was lower, while the metestrus and diestrus indices of the glyphosate-based herbicide-treated groups were lower than the control group (Table 2).

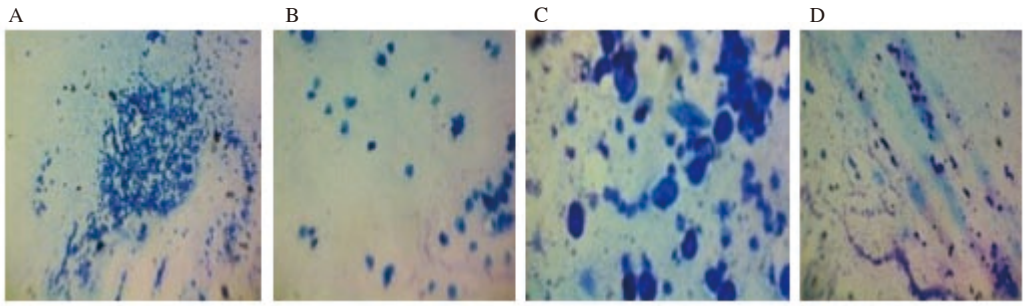

Figure 1. Photomicrograph of vagina cytology of rat shows (A) proestrus with small round nucleated cells with deep basophils in clusters, (B) estrus with anucleated keratinized epithelial cells, (C) metestrus with a combination of anucleated cells and neutrophils, and (D) diestrus with few anucleated cells but mostly neutrophils with small and large nucleated cells with mucous.

Table 1. Stage of oestrous cycle of rats administered glyphosate-based herbicide for five cycles.

Stage of oestrous cycle, days	Control	250 mg/kg	500 mg/kg
Proestrus	5.0±0.0[a]	6.2±0.4[b]	4.2±1.1[a]
Estrus	5.0±0.0[a]	9.2±0.4[b]	11.6±1.1[c]
Metestrus	5.0±0.0[a]	1.6±0.5[b]	1.8±0.4[c]
Diestrus	5.0±0.0[a]	3.0±0.0[b]	2.4±0.5[c]

Data are expressed as mean±SD. Rows with different superscripts are statistically significant with $P<0.05$.

Table 2. Oestrous cycle index and ratio of rats administered glyphosate-based herbicide for five cycles.

Oestrous cycle index	Control	250 mg/kg	500 mg/kg
Proestrus index	25	31	21
Estrus index	25	46	58
Metestrus index	25	8	9
Diestrus index	25	15	12
Oestrous cycle ratio	1.0±0.0[a]	3.4±0.6[b]	3.9±1.1[b]

Data are expressed as mean±SD or number. Rows with different superscripts are statistically significant with $P<0.05$.

Table 3. Length of oestrous cycle of rats administered glyphosate-based herbicide for five cycles.

Length of oestrous cycle	Control	250 mg/kg	500 mg/kg
Pre-administration	4.0±0.0	4.0±0.0	4.0±0.0
First cycle	4.0±0.0[a]	3.6±0.6	3.2±0.5[b]
Second cycle	4.0±0.0	3.8±1.9	2.8±1.1
Third cycle	4.0±0.0[a]	1.8±0.8[b]	1.2±0.5[b]
Fourth cycle	4.0±0.0[a]	1.8±0.8[b]	1.4±0.2[b]
Fifth cycle	4.0±0.0[a]	1.8±0.4[b]	1.2±0.2[b]

Data are expressed as mean±SD. Rows with different superscripts are statistically significant with $P<0.05$.

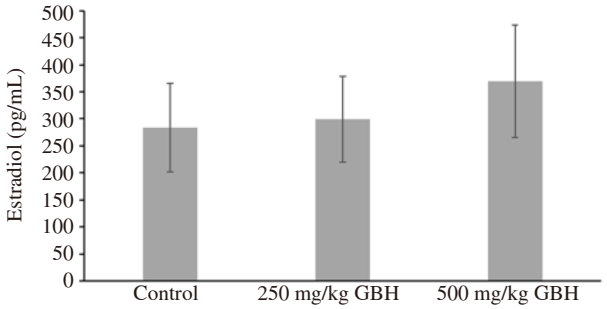

Figure 2. Estradiol level of rats administered glyphosate-based herbicide (GBH) for five cycles.

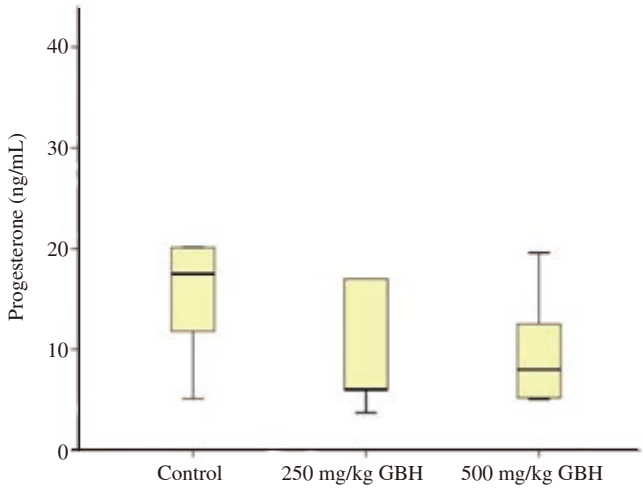

Figure 3. Progesterone level of rats administered glyphosate-based herbicide (GBH) for five cycles.

3.3. Oestrous ratio

The oestrous cycle ratio is also presented in Table 2. There was a significant increase in the oestrous cycle ratio of the glyphosate-based herbicide-treated groups compared to the control group ($P<0.05$).

3.4. Length of oestrous cycle

The mean length of oestrous cycle is presented in Table 3. There was a decrease in the length of oestrous cycle in the glyphosate-based herbicide-treated groups compared to the control group in the first cycle, although the decrease was only significantly different between the 500 mg/kg group and the control group ($P<0.05$). During the second cycle, there was no significant difference between the glyphosate-based herbicide-treated groups and the control group ($P>0.05$). However, there was a significant decrease between the glyphosate-based herbicide-treated groups and the control group in the third, fourth and fifth cycles ($P<0.05$).

3.5. Estradiol and progesterone levels

The mean estradiol and progesterone levels are shown in Figures 2 and 3, respectively. There was a dose-dependent increase in the estradiol level of the glyphosate-based herbicide-treated groups compared to the control group, although this was not statistically significant (P>0.05). Similarly, there was an insignificant dose-dependent decrease in the progesterone level of the glyphosate-based herbicide-treated groups compared to the control group (P>0.05).

4. Discussion

Glyphosate is the active ingredient in most herbicide-based glyphosate used worldwide[18]. In the present study, the proestrus stage increased in the 250 mg/kg group as well as the oestrus stage of all the glyphosate-based herbicide-treated groups. This is similar to the report of Schimpf *et al*[19] in rats administered glyphosate-based herbicide postnatally for 7 days. It is also similar to the report of Mustapha *et al*[20], Abu and Uchendu[21] and Essiet *et al*[22] in rats administered extracts of *Rhynchosia sublobata*, *Hymecocardia acida* and *Salacia lehmbachii*, respectively. It differs from the report of Rahman *et al*[23] in rats, Baligar and Kaliwal[16] in mice, Auta and Hassan[24] in *Mus musculus* administered *Eurycoma longifolia*, carbofuran and *Azadirachta indica*, respectively. The increase in the present study may be attributed to the ability of glyphosate-based herbicide to either bind to estrogen receptors[25,26] or mimic the effect of estrogen[11]. Once the estrogen receptors are blocked or mimicked, it can lead to an increase in estrogen in the blood, which is the hormone that predominates during the oestrus and proestrus stages of the cycle[27]. The metestrus and diestrus stages decreased in our study, which is similar to the reports of Mustapha *et al*[20] in rats administered extracts of *Rhynchosia sublobata*. However, it is contrary to the reports of Abu and Uchendu[21], Rahman *et al*[23], Essiet *et al*[22] and Chika *et al*[28] in rats given *Hymecocardia acida*, *Eurycoma longifolia*, *Salacia lehmbachii* and *Adansonia digitata*, respectively. It is also contrary to the reports of Auta and Hassan[24] in *Mus musculus* administered *Azadirachta indica*. Baligar and Kaliwal[16], reported increased metestrus similar to our report but observed an increase in diestrus. The estrogenic effect of glyphosate-based herbicides may be responsible for this. Progesterone is the dominant hormone during the metestrus and diestrus stages, and this hormone acts in synergy with estrogen to control the oestrous cycle[29].

The oestrous cycle indices in this study were altered following glyphosate-based herbicide administration. This was characterized by a decrease in diestrus and metestrus indexes, as well as an increase in proestrus and oestrus indexes. This is similar to an earlier report in rats exposed to dicofol[30]. The result of the present study is different from the report of Ganesan *et al*[31] in mice exposed to 2 mg/kg glyphosate for 5-10 weeks, where no meaningful change was observed in the time spent at any stage of the oestrous cycle, though there was a tendency for increased time spent in oestrus and reduced time in metestrus/diestrus after 5 weeks of glyphosate exposure. This may be associated with the low dose of glyphosate-based herbicide used in that study. The present study is also different from the report of Baligar and Kaliwal[16], who reported a decrease in the proestrus, oestrus, and metestrus indices with an increase in the diestrus index in rats following carbamate administration. There was a decrease in the length of the oestrous cycle in the rats administered glyphosate-based herbicide in the present study. This is contrary to the reports of Schimpf *et al*[19] and Essiet *et al*[22] in rats administered 2 mg glyphosate-based herbicides and *Salacia lehmbachii*, respectively, where an increase was observed. The high dose of glyphosate-based herbicide used in the present study may be responsible for this variation because the length of the oestrous cycle began to reduce from the first cycle in the 500 mg/kg glyphosate-based herbicide group and from the third cycle in the 250 mg/kg group. In addition, the persistent proestrus and oestrus in the glyphosate-based herbicide-treated groups were probably caused by the glyphosate-induced increase in estrogen. This may account for the decrease in the length of the oestrous cycle. A similar decrease in the length of the oestrous cycle has been reported in rats administered dicofol[30], mice administered carbofuran[16], and goats infected with *Trypanosoma congolense*[32]. It is important to note that any alteration in the length of the oestrous cycle is capable of causing infertility in an animal[24].

There was an increase in the oestrous cycle ratio of the glyphosate-based herbicide group in the present study. The oestrous cycle ratio is the proportion of proestrus and oestrus to the proportion of metestrus and diestrus[33]. An increase in the oestrous cycle ratio indicates a persistent follicular phase characterized by prolonged proestrus and oestrus stages[16]. These stages increased in our study as earlier stated and may not be unconnected with the ability of glyphosate-based herbicides to bind estrogen receptors[26]. While estradiol was increasing, progesterone was decreasing with increasing doses of glyphosate-based herbicides. A similar observation was reported by Ren *et al*[34] and Schimpf *et al*[19] in rats administered glyphosate-based herbicide, but different from the reports of Manservisi *et al*[35] in rats and Ganesan *et al*[31] in mice administered glyphosate-based herbicide. The difference may probably be due to the very low dose used in those studies compared to the present study. This suggests that at a higher dose, glyphosate-based herbicide was likely to cause alterations in estradiol and progesterone levels. When estradiol level is maintained at a tonic level, it can result in persistent oestrus[36,37]. The prolonged periods of oestrus in this study may be responsible for this. Low levels of progesterone are considered responsible for the maintenance of oestrus[38]. The persistent proestrus is also a result of an increase in estrogen levels but not high enough to move to the oestrus stage[36]. Persistent oestrus is usually followed by pseudopregnancy[38]. This period is characterized by an active corpora lutea[38]. The high estrogen level and low progesterone

level in the study may be responsible for the persistent proestrus and oestrus reported earlier in this study. This persistence may be the cause of the decrease in the length of the oestrous cycle and an increase in the proestrus and oestrus indices.

This study was able to evaluate the effect of a glyphosate-based herbicide on the oestrous cycle of Wistar rats. However, a major limitation of the study was our inability to determine the daily and weekly patterns of estradiol and progesterone. We, therefore, recommend further studies in which these hormones will be assessed more frequently as this will provide further insights into the extent of effect glyphosate-based herbicide has on the endocrine glands

In conclusion, the study reveals a dose-dependent alteration of the oestrous cycle of rats administered glyphosate-based herbicide. This is characterized by changes in the oestrus and oestrous cycle index. In addition, there is a decrease in the length of the oestrous cycle and an increase in the oestrous cycle ratio. It is therefore recommended that farmers be educated on the adverse effect of glyphosate-based herbicide on reproduction to guide against indiscriminate use.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

Hafsat Yazeed Idris and Adewale Ayodeji Adeyeye conceived and designed the study. Hafsat Yazeed Idris, Adewale Ayodeji Adeyeye and Emmanuel Busayo Ibitoye participated in the data processing. Hafsat Yazeed Idris wrote the initial draft, while Adewale Ayodeji Adeyeye, Emmanuel Busayo Ibitoye and Millicent Ladi Umaru critically reviewed and edited the final draft.

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