Genotoxic Studies Of Roundup And Stomp Herbicides On Nile Catfish (*Clarias Gariepinus*)

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ABSTRACT

The present study was designed to evaluate the genotoxic effects of Roundup, Stomp herbicides and their combination on Nile catfish (*Clarias gariepinus*). The experiment was carried out on 120 fish that randomly divided into four equal groups with three replicates; the first group kept as control, the second group exposed to 1/2 96 hrs LC_{50} of roundup, the third group exposed to 1/2 96 hrs LC_{50} of Stomp and the fourth one exposed to a combination of roundup and Stomp at the doses previously mentioned. The experiment was terminated after 15 days where The sera were separated for estimation of 8-hydroxy 2-deoxy guanisine (8-OHdG) then the fish were sacrificed and specimens from gills of all groups were obtained and kept at -20°C for comet assay and another specimens from the same organ were fixed in 10% neutral – buffered formalin for histopathological examination. The results indicated that both herbicides individually cause significant increase in DNA damage that was highly obvious in group exposed to the combination of both herbicides. Histopathological investigation of gills confirmed the aforementioned findings.

INTRODUCTION

Using agrichemicals to control unwanted species has become a necessary and common world wide practice to improve crop production. Although most currently used agrichemicals are considered relatively safe, continuous usage contributes contamination for soil and water and collateral toxic effects on aquatic species *(1)*.

Pesticides have become some of the most frequently occurring organic pollutants of agricultural soils, ground and surface waters, causing ecological imbalances *(2)* that may have toxicological effects on natural ecosystems, especially aquatic system *(3)*. They cause damage to non-target organisms, including fish *(4)*.

Glyphosate is a broad-spectrum non-selective herbicide used for inhibition of unwanted weeds and grasses in agricultural, industrial, urban, forestry and aquatic landscapes *(5)*.

Recent studies (mainly from 2000 onward) are showing potentially adverse effects of Roundup, and its components glyphosate and polyoxyethylene amine (POEA) on fish. For example, Roundup affected energy metabolism, free radical processes, and acetylcholine esterase activity *(6)*.

Glyphosate treatment of human lymphocytes *in vitro* resulted in increased chromatid exchange *(7)*, chromosomal aberrations and indicators of oxidative stress *(8)*. Furthermore, Roundup proved to increase DNA adducts in mice *(9)*. Recently, comet assay was used to measure glyphosate impact

on DNA of human lymphocytes and the results revealed that glyphosate increased tail intensity *(10)*.

Stomp is liquid emulsive herbicide of the dinitroaniline type; its active ingredient is pendimethalin *(11)*. Pendimethalin is a selective herbicide used to control most annual grasses and certain broadleaf weeds in field corn, potatoes, rice, cotton, soybeans, tobacco, peanuts, and sunflowers *(12)*.

The use of herbicide mixtures for weed control has been proposed as one of the most cost-effective strategies *(13)*. However, this strategy runs contrary to the views of many environmental scientists because chemical mixtures in the environment may lead to additive, synergistic or antagonistic effects on target organisms, and they thus pose a greater potential threat to environmental safety and human health than the individual chemicals *(14)*.

Although there is a considerable amount of information available on toxicity of individual herbicides to fish and aquatic invertebrates, there is less information on toxicity of herbicide mixtures to these organisms. Also the impacts of Stomp on fish have so far undergone little research and there is still a great need to properly assess the impact of Stomp on fish. Therefore, the present work was intended to shed some light on the genotoxic effects of Roundup and Stomp and their combination on Nile catfish (*Clarias gariepinus*).

MATERIAL AND METHODS

Fish and experimental protocol

A total number of 120 Nile catfish (*Clarias gariepinus*) with a body weight ranged from 90- 115 gm were used. fish were obtained from the ponds of the Central laboratory of Aquaculture Researsh, Abbassa, Abo-Hammad, Sharkia, Egypt. Fish were apparently healthy and free from skin lesions or external parasites, they were maintained in glass aquaria (50x40x150 cm capacity) having 180 litres of dechlorinated tap water. Each aquarium provided with aeriator, thermostatically controlled with heater and thermometer. Fish were acclimatized for two weeks to laboratory environment. Fish were fed 3 times daily on a basal diet contained 35.4 % crude protein. The amount of food per day was 3 % of fish body weight. The experiment was conducted for two weeks

The fish were divided into four equal groups with three replicates, the first group kept as control, the second group exposed to 1/2 96 hrs LC_{50} of roundup (14.5 mg/L) *(15)* while, the third one exposed to 1/2 96 hrs LC_{50} of Stomp (420 µg/L) *(16)*. In the fourth group, the fish were exposed to a combination of 1/2 96 hrs LC_{50} of both of roundup and Stomp.

Tested compounds

1-Roundup (a glyphosate-based herbicide): It was obtained in commercial form (Roundup) containing 48% EC (emulsion concentration). It was obtained from Monsanto Agriculture Company, USA.

Empirical Formula: $C_3H_8NO_5P$

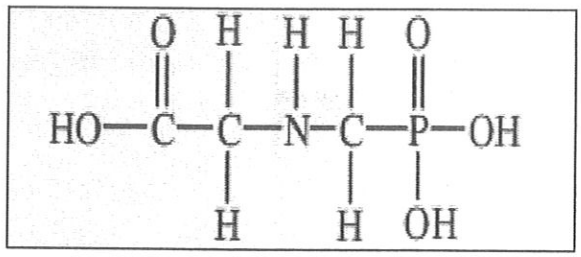

Fig. 1. Chemical structure of Glyphosate *(17)*.

2- Stomp: Stomp® 50% EC (BASF PLC) is an orange-yellow liquid emulsive herbicide of the dinitroaniline type *(18)* its active ingredient is pendimethalin *(11)*. Stomp contains the inert components (50%); as petroleum solvents (naphthalene and ethylene dichloride). Empirical Formula: $C_{13}H_{19}N_3O$.

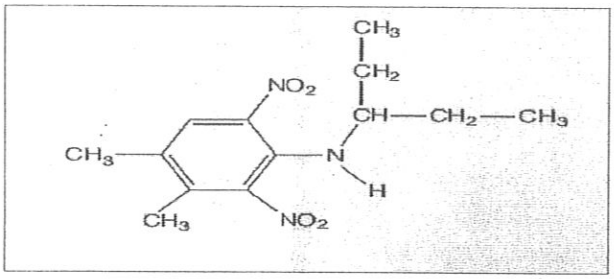

Fig. 2. Chemical structure of Pendimethalin *(19)*.

Sampling and measurments

At the end of the experimental period (2 weeks), blood samples were collected from the caudal blood vessel *(20)* using sterile syringes, left to clot at room temperature followed by centrifugation at 3000 r.p.m. for 15 minutes for serum separation, then fish were sacrificed by decapitation and dissected. Specimens from gills of the treated and control groups were obtained and kept at -20°C for comet assay. Another specimens from the same organ of all groups preserved in 10% neutral –buffered formalin for histopathological examination.

Genotoxic studies

1) Alkaline single cell gel electrophoresis (comet assay)

   The alkaline comet assay was performed *(21)*.

2) Estimation of serum 8-hydroxy-2'-deoxyguanosine (8-OH2'dG)

It was estimated by enzyme linked immunosorbent assay (ELISA) using Cayman Chemical's ACETM EIA kits, from Cayman Chemicals Company, Ann. Arbor, MI. USA.

Histopathological studies

Histopathological examination of gills of tested *Clarias gariepinus* was carried out *(22)* and then examined microscopically.

III- Statistical analysis

The obtained data were analyzed and graphically represented using the statistical package for social science *(23)* for obtaining mean and standard error. The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups.

RESULTS

Alkaline single cell gel electrophoresis (comet assay)

Regarding the oxidative DNA damage caused by both herbicides, the present study revealed that exposure to Roundup and/or Stomp caused an increase in DNA damage in gills of *Clarias gariepinus* indicated by the damaged nuclei (tail DNA %, tail length and tail moment) as the exposure to Roundup /or Stomp individually induced a significant increase in tail DNA %, tail length and tail moment compared with the control group. On the other hand, fish exposed to the combination of both herbicides (Roundup and Stomp) showed an obvious significant increase in % of DNA damage and a highly significant increase in tail length and tail moment compared with the control group (Table 1 and Figs 3-4).

Table 1. The oxidative DNA damage (comet assay) in gills of *Clarias gariepinus* exposed to Roundup, Stomp and both after 15 days of exposure (Mean± S.E.) (n=30)

Parameters \ Groups	- ve control	1/2 LC_{50} Roundup	1/2 LC_{50} Stomp	1/2 LC_{50} Roundup & 1/2 LC_{50} Stomp
Tail length	6.92± 0.41^{a}	10.0± 0.50^{bc}	7.58± 0.52^{ab}	12.31± 1.40^{c}
Tail DNA %	0.73± 0.42^{a}	3.56± 0.54^{b}	3.23± 0.11^{b}	4.05± 0.47^{b}
Tail moment	0.08± 0.04^{a}	0.3± 0.02^{b}	0.22± 0.03^{b}	0.47± 0.02^{c}

Means within the same row having the different superscript were significantly different (P< 0.05)

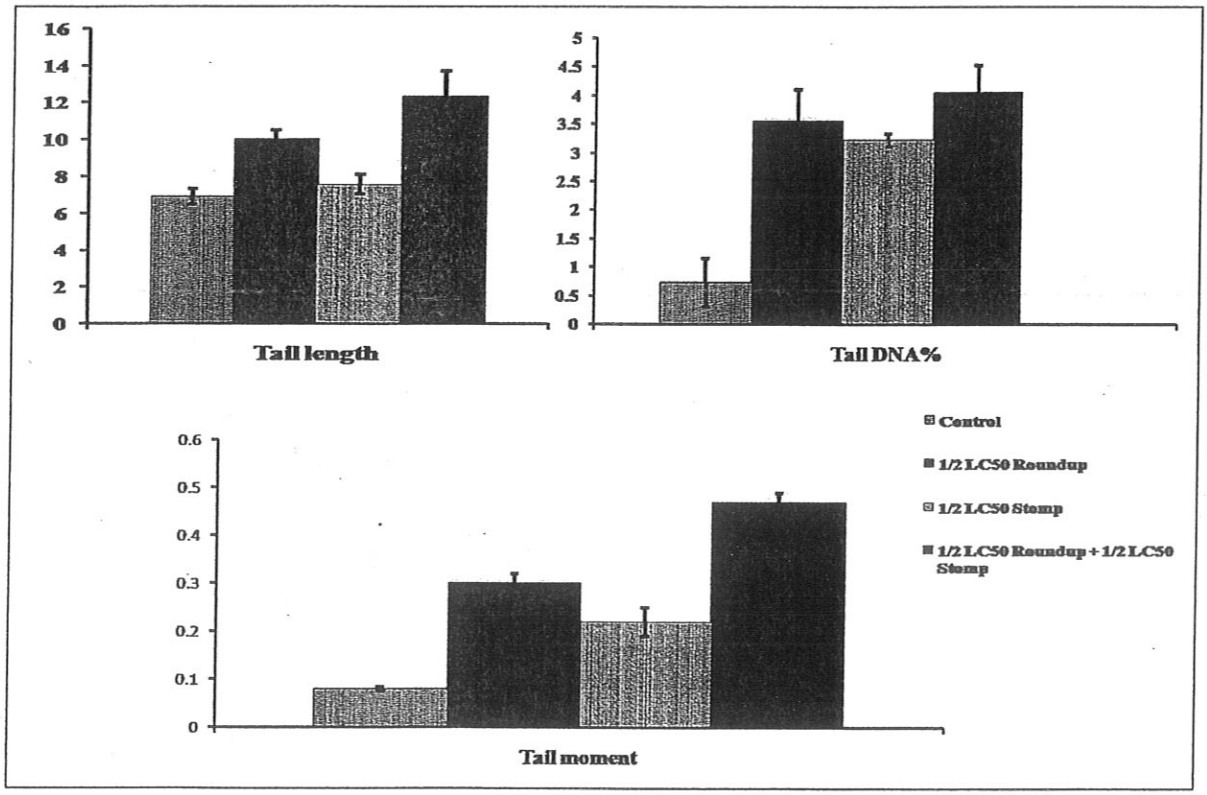

Fig. 3. Changes in tail DNA %, tail length and tail moment in gills cells of *Clarias gariepinus* exposed to Roundup, Stomp and both after 15 days of exposure (Mean± S.E.). (n=30).

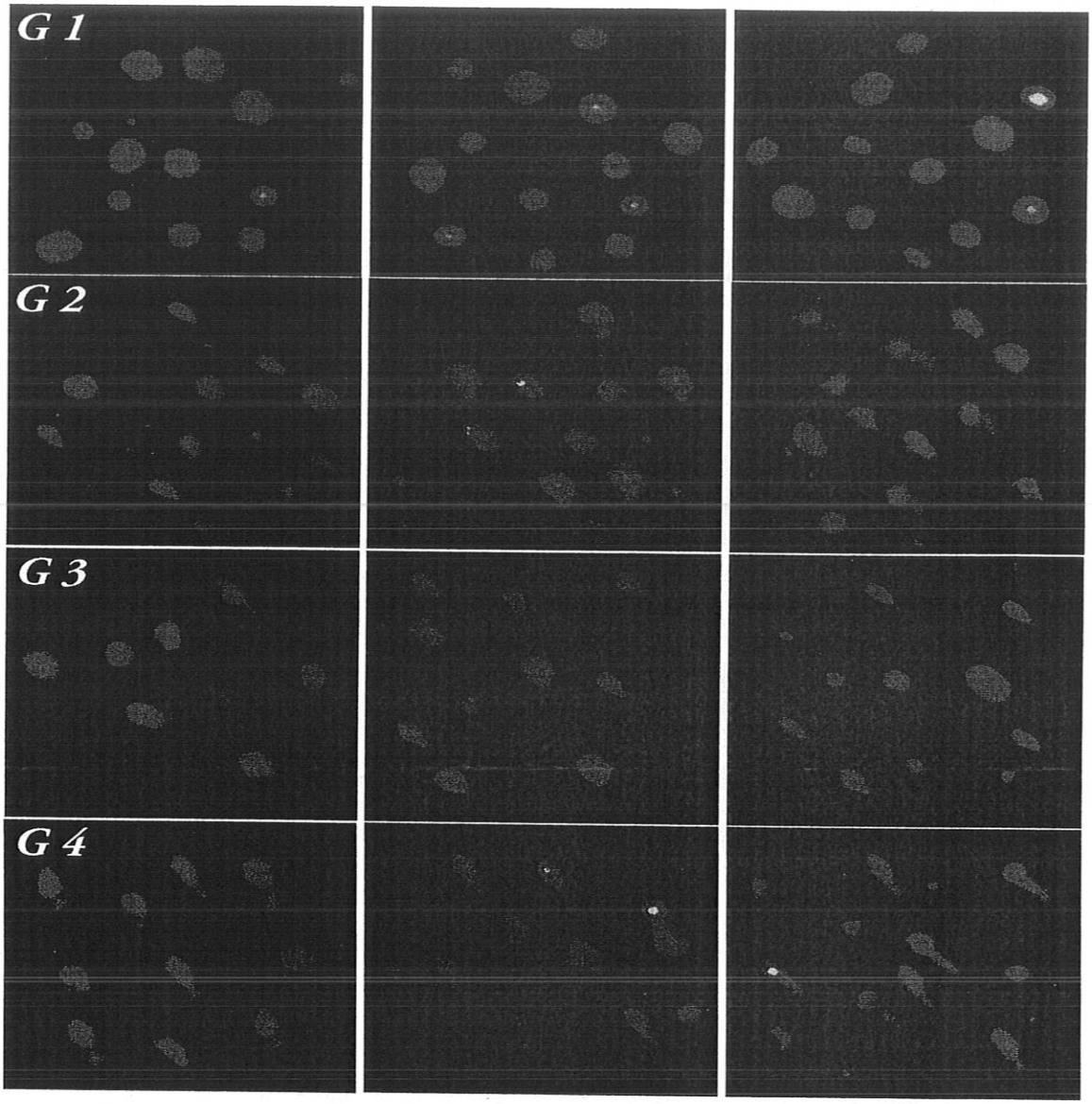

Fig.4. Photomicrograph with florescent microscope showing the effect of Roundup, Stomp and both on oxidative DNA damage of gills cells of *Clarias gariepinus* after 15 days of exposure

G1) Control group: almost normal condensed type nucleus

G2) Group exposed to 1/2 LC_{50} of roundup (14.5 mg/L).

G3) Group exposed to 1/2 LC_{50} of Stomp (420 µg/L).

G4) Group exposed to1/2 LC_{50} of roundup + 1/2 LC_{50} of Stomp showing cells with damaged DNA appeared as a comet

Estimation of serum 8-hydroxy-2'-deoxyguanosine (8-OH2'dG)

Concerning the serum level of 8OH2\dG, *Clarias gariepinus* exposed to Roundup or Stomp separately showed a significant elevation in 8OH2\dG level while, there was an obvious significant elevation in the level of 8OH2\dG in group treated with the combination of both herbicides comparing with the control group as shown in table 2 and fig.5.

Table 2.Changes in 8OH2\dG (ng/ ml) level in serum of *Clarias gariepinus* exposed to Roundup, Stomp or Roundup plus Stomp at 15th day of exposure (Mean± S.E.) (n=30)

Parameters \ Groups	- ve control	1/2 LC_{50} Roundup	1/2 LC_{50} Stomp	1/2 LC_{50} Roundup & 1/2 LC_{50} Stomp
8OH2\dG (ng/ ml)	3.57± 0.40 [a]	7.23± 1.06 [b]	6.84± 0.07 [b]	13.44± 1.07 [c]

Means within the row having the different superscript were significantly different (P< 0.05)

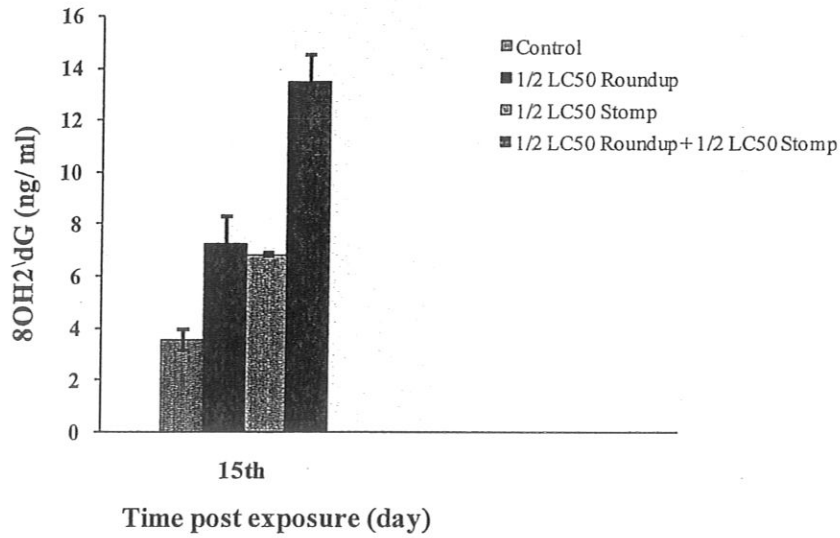

Fig. 5. Changes in 8OH2\dG level in serum of *Clarias gariepinus* exposed to Roundup, Stomp and both after 15 days of exposure (Mean± S.E.) (n=30)

Histopathological findings

Examination of gills of *Clarias gariepinus* in all treated groups at the end of experiment revealed many lesions.

The gills of control showed normal filaments and covering epithelim **(Fig 6A)**. The gills of group exposed to Round up showed congestion in central sinuses, packed with many erythrocytes as well as hemorrhage in the central areas. Proliferations of the respiratory epithelia were seen besides many necrotic changes and lymphoid cells infiltration **(Fig 6B)**. The majority of these epithelia were swollen and induced lamellar fusion **(Fig 6C).** While, the gills in group exposed to Stomp showed intense aggregation of mononuclears on necrotic filaments and slight congestion **(Fig 6D)**. Many putative lymphocytes were seen infiltrating the interlamellar epithelium which consisted of proliferated and necrotic cells. The gill rackers revealed severe desquamation and mucinous degeneration in the covering epithelium besides numerous eosinophil granular cells (EGCs) infiltrations **(Fig 6E).** Finally, the gills in group treated with both Roundup and Stomp showed severe congestion, clubbing of gill filaments and proliferation of the interlamellar epithelia **(Fig 6F)**. Extensive necrosis, lymphoid cells infiltration and proliferation of mucous cellswere visualized **(Fig 6G).** The gill rackers showed mucinous degeneration and leukocytes infiltrations besides congestion and edema in the lamina propria **(Fig 6H)**.

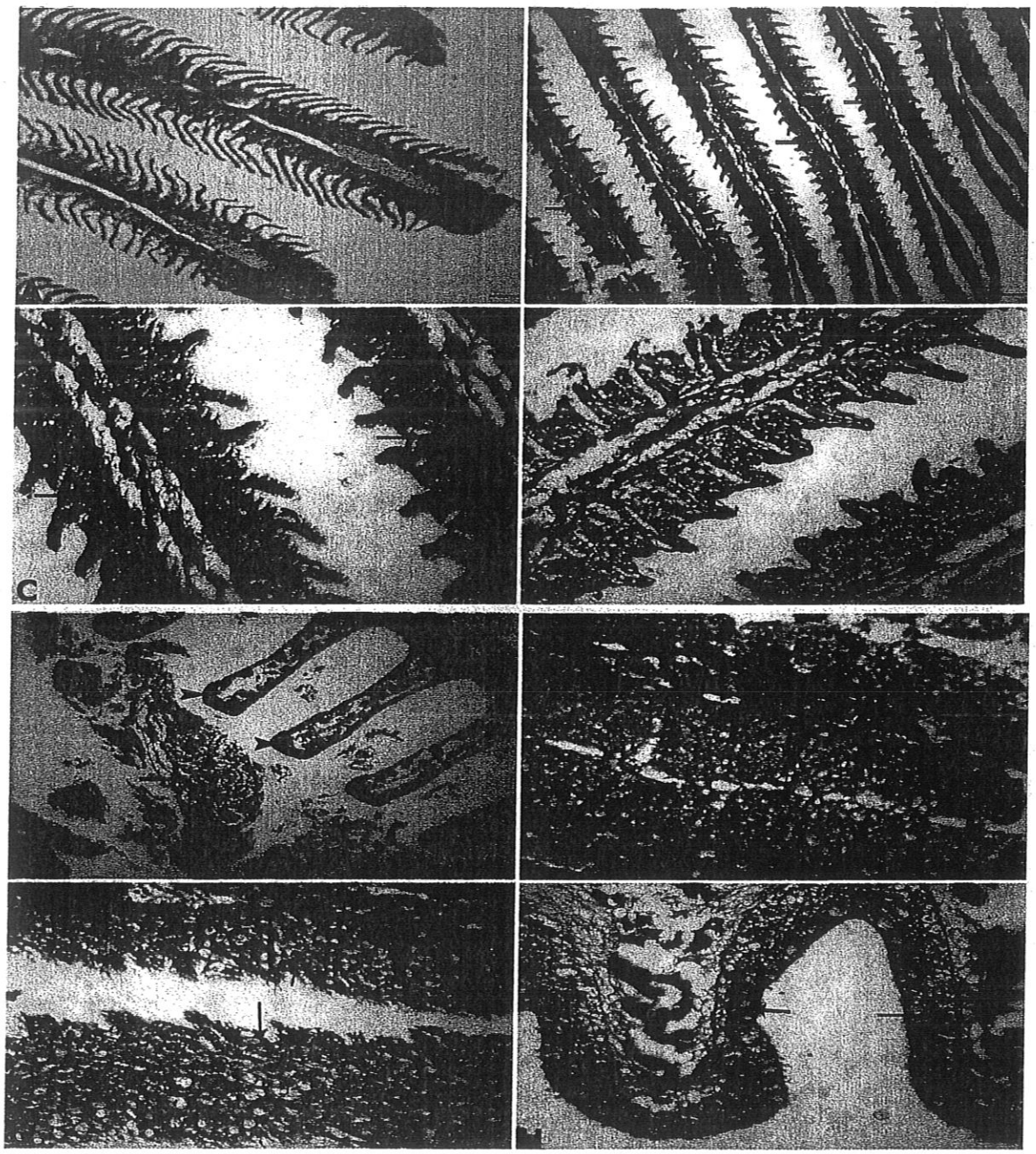

Fig 6. Photomicrograph of gills from different groups: Control shows normal filaments and covering epithelim **(A)**. Round up shows congestion in central sinuses, proliferation of respiratory epithelia and some necrotic changes and lymphoid cells infiltration (arrows) **(B)** and a higher magnification of previous fig **(B).** with lamellar fusion (arrows) **(C).** Stomp shows intense aggregation of mononuclears on necrotic filaments and congestion (arrows) **(D)** and severe desquamation (arrow) and mucinous degeneration (arrowheads) in the covering epithelium of gill rackers **(E)**. Both Roundup and Stomp shows severe congestion (arrowhead), clubbing of gill filaments and proliferation of the interlamellar epithelia (arrows) **(F),** necrosis, lymphoid cells infiltration (arrows) and proliferation of mucous cells (arrowheads) **(G)** and mucinous degeneration (arrows) and leukocytes infiltrations on the gill rackers **(H). HE x Scale bar = 25 µm.**

DISCUSSION

Genotoxic studies in fish are frequently performed in erythrocytes, due to the ease of sampling and their adaptability to the most common methodologies *(24)*. However other cell types should be used for monitoring genotoxic effects *(25)* , thereby exploiting tissue-specific responses and acquiring a better perspective about the overall condition of the organisms. When water born contamination is considered, gills are the first target organ due to the large surface area in direct and continuous contact with the external medium, and its involvement in uptake *(26)*. Morever, fish gills can be more vulnerable towards oxidative damage than other organs (e.g. liver) and may respond earlier to a pollutant-induced pro-oxidant challenge *(27)*.

The present study elucidate the genotoxicity of Roundup and Stomp by using the alkaline SCGE (comet assay) to evaluate total DNA strand breaks in gills cells of freshwater fish *Clarias gariepinus*. The results of our study showed that exposure of *Clarias gariepinus* to Roundup and/or Stomp induced significant increase DNA damage via increased tail length, tail moment and tail DNA% in gills cells comparing with the control group which indicated the genotoxic potential of the both herbicides to aquatic organisms. The DNA damage as observed in the present study is in consistent with previous findings on the blood cells of gold fish (*Carassuis auratus*) after exposure to 5, 10 and 15 mg/L of glyphosate *(5)* and on the blood and gills cells of *Prochilodus lineatus* exposed to 10 mg/L of the same herbicide *(28)*. Also these results were in accordance with other study which revealed that exposure to 5% and 10% 96 hrs LC_{50} of stomp (Pendimethalin) induced DNA damage in liver and gills cells of *Oreochromis niloticus (29)*.

The DNA damage detected in the present study could have originated from DNA single-strand breaks, DNA double-strand breaks, DNA–DNA/DNA-protein cross-linking or inhibition of the enzymes involved in DNA repair resulting from the interaction of the herbicide, its metabolites or ROS generated by the metabolism of the herbicide with DNA of exposed fish resulting in the lesions detected by comet assay *(30)*. It is also could be explained by the direct toxic and inhibitory effect induced by the examined herbicides *(31)*, or by inhibition of DNA repair during the exposure time *(5)*. Finally, numerous xenobiotics, including pesticides, can produce reactive oxygen species (ROS) via several mechanisms, e.g., inactivation of antioxidant enzymes, depletion of nonenzymatic antioxidants, and membrane lipid peroxidation *(32)*. Increased levels of ROS may result in DNA oxidation and elevated steady-state levels of unrepaired DNA resulting in an even higher negative impact into DNA *(33)*. Hence, under these circumstances, incidence of DNA damage is increased in gills cells of the fish. Thus, it is possible that herbicides could have caused alterations in DNA of *Clarias gariepinus* resulting in formation of comets.

8oH2\dG is one of the major forms of oxidative DNA damage and has been well studied because it is relatively easy to be detected and can be assayed in biological fluids such as serum, saliva and urine. Currently, the influence of oxidative stress that occurs in various disorders is estimated using a reactive oxygen species (ROS) modulator, active oxygen erasing system enzymatic antioxidants, or genetic transcription factors. To determine the site of DNA damage, 8oH2\dG (the most popular biomarker) level was assessed in serum of *Clarias gariepinus* exposed to Roundup and/or Stomp, after it has been investigated the DNA damage by SCGE technique. Our study revealed a significant increase in the level of 8oH2\dG due to the ability of ROS to induce DNA damage via causing DNA-protein cross links and modifications of base residues such as introduction of a hydroxyl group (-OH) into the C-8 position of guanosine and guanine residues forming 8-OHdG and of DNA oxidation *(34,35)*. These data suggest that 8oH2\dG is a useful biomarker of oxidative

stress and DNA damage which confirm previous investigations *(36, 37)*.

The aforementioned picture of the present study regarding DNA damage in gills came in harmony and confirmed by histopathological changes in *Clarias gariepinus*, The more frequent changes in the gills of *Clarias gariepinus* exposed to Roundup and/or Stomp were intense aggregation of mononuclears on necrotic filaments and slight congestion. Many putative lymphocytes were seen infiltrating the interlamellar epithelium which consisted of proliferated and necrotic cells. The gill rackers revealed severe desquamation and mucinous degeneration in the covering epithelium besides numerous eosinophil granular cells infiltrations. These findings are supported by previous studies *(38, 39)* which reported that herbicides not only enter the organism through the gills, but also exert its primary toxic effects on the branchial epithelium and may influence the general gills functions.

Conclusion: The both herbicides, is water pollutant and caused genotoxicity to *Clarias gariepinus*. Toxicity can end up in humans through the food chain. The suitable control, and regular use of herbicides is recommended in order to obtain the beneficial effects of these resources without polluting the environment and without leaving their residues in food and water sources with potentially negative effects on human health

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الملخـــص العربـــي

دراسة التأثير السمى الجينى لمبيدى الحشائش الراوند أب والاستومب على سمك القرموط النيلى

فوزي عيد شعبان ،على حيدر عبد الرحمن ابو حديد ،جيهان جمال السيد مصطفى ، ولاء محمد الهادى
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تستهدف هذه الدراسة الى تقييم التأثير السمى الجينى لمبيدى الحشائش الراوندأب والاستومب على سمك القرموط النيلى. استخدم ١٢٠ سمكة وقد تم تقسيم هذه الأسماك الى أربع مجموعات متساوية. استخدمت المجموعة الأولى كضابط للتجربة أما المجموعة الثانية فقد تم تعريضها الى نصف التركيز المميت لمبيد الحشائش الراوند أب (١٤,٥ ميللى جرام/ لتر) ، المجموعة الثالثة تم تعريضها الى نصف التركيز المميت لمبيد الحشائش ستومب (٤٢٠ ميكروجرام /لتر) أما المجموعة الرابعة فقد تم تعريضها لكلا المبيدين معا كما هو مبين فى المجموعتين الثانية والثالثة وذلك لمدة ١٥ يوما. وبعد انهاء مدة التجربة تم الحصول على عينات دم وفصلها للحصول على مصل الدم وحفظه عند درجة حرارة -٢٠ درجة سيليزية لقياس مستوى ٨ هيدروكسي ٢ ديؤكسي جوانيزين. واخذت عينات من الخياشيم وتم حفظها عند درجة حرارة -٢٠ درجة سيليزية لدراسة التغيرات الوراثية فى الحمض النووى الديؤكسى ريبوزى بواسطة اختبار المذنبات. أما الجزء الآخر من الخياشيم فقد تم حفظها فى الفورمالين المتعادل لدراسة التغيرات الهستوباثولوجية. وأسفرت النتائج أن كلا من المبيدين أدى الى احداث تلف وتدميرفى الجمض النووى الديؤكسى ريبوزى وأيضا زيادة واضحة فى مستوى ٨ هيدروكسي ٢ ديؤكسي جوانوزين ولوحظ أن هذه الزيادة كانت أكثر وضوحا فى المجموعة المعرضة لكلا المبيدين معا. بالاضافة الى زيادة فى التغيرات الهستوباثولوجية والتى اظهرت تغيرات واضحة فى انسجة الخياشيم والتى تتمثل فى احتقان شديد فى الأوعية الدموية ووجدت بعض الارتشاحات لبعض الخلايا المستديرة فى النسيج الابثيليومى بالاضافة الى انتفاخ بعض خلايا الغشية المبطنة لها بالاضافة الى العديد من المناطق المحتقنة مع وجود نزيف دموى بين الخلايا.