Biochemistry Letters, 13(25) 2018, pages(313-328)

Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Journal home page:

Potential Effects of Cadmium Chloride and Glyphosate on DNA Damage in Nile tilapia

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Key words

Nile tilapia, Glyphosate, Cadmium chloride, Molecular biomarker, Comet assay, DNA damage.

ABSTRACT

The present study was conducted to estimate the effect of cadmium chloride as a heavy metal and commercial glyphosate (Roundup®) as a herbicide on DNA damage as biochemical and molecular biomarkers in the Nile tilapia, *Oreochromis niloticus* through three exposure periods with different concentrations of LC_{50}. The 96h-LC_{50} were determined for $CdCl_2$ (132mg/l), glyphosate (9.63mg/l), $CdCl_2$ in mixture (41.30mg/l) and glyphosate in mixture (2.75mg/l), respectively. The fish exposed to these concentrations separately and mixed for 4 days as well as two sublethal concentrations (1/4 and 1/10 LC_{50}) for 8 days and 45 days, respectively. Gills and liver cells of the exposed samples were taken after 4, 8 and 45 days to examine the DNA damage using comet assay. The highest DNA damage was observed on gills after 4 days in treatment of mixture due to presence of synergistic effect between $CdCl_2$ and glyphosate , after 8 days in treatment of glyphosate and after 45 days in treatment of cadmium. The highest DNA damage was observed on liver after 4days and 8days in treatment of glyphosate but after 45days in treatments of cadmium and mixture. DNA damage depends on concentration of pollutants and exposure periods.

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Introduction

Fish is the main and cheap food source for humans due to its high mineral and protein content with low-fat residue. It is available in different species, sizes, and ages which is preferred in toxicological research [1]. Fish is the animal protein source in many developing countries [2]. Nile tilapia is the most important aquaculture fish species in Egypt. It is representing in more than 80% of total tilapias production [3]. Egypt is the second in cultured tilapia's production directly after China [4]. Fish plays an important role in monitoring heavy metals contamination as biomarker [5].

Bioaccumulation of heavy metals in aquatic organisms causes strong threat to health due to chronic exposure to high concentrations [6,7]. All laboratory studies confirmed that the accumulation of heavy metals in tissues and cells of aquatic organisms depends on the period of exposure, concentration of metals, water quality, factors of temperature, oxygen concentration, hardness, hydrogen ion concentration, salinity, alkalinity and dissolved organic carbon [8,9,10].

Cadmium is a heavy element that is easily soluble in water as it is absorbed by aquatic organisms directly from water. It is accumulated at high concentrations in the liver tissue of the fish causing various pathological changes in liver tissues [11]. Gills are also represented the store house of cadmium in experimental studies [12]. Cadmium accumulated in major organ tissues of fish like liver, stomach and gills [13].

Glyphosate,[*N*-(phosphoromethyl)glycine] from herbicides was used in agricultural fields to eliminate annual weeds, broad-leafed grasses and woodyshrubs [14]. Polyoxyethylene amine (POEA) substance was used in formulation of Glyphosate to become very toxic in commercial formula called Roundup® [15]. It reaches the aquatic environment through agricultural crops spread along the edges of canals and rivers. So, fish is bioindicator of water pollution with herbicides, especially glyphosate [16]. Glyphosate as herbicide is widely used with different trade names and formulations [17].

DNA damage was used as biomarker of the genotoxicity of toxic agents to aquatic organisms as fish [18]. Numerous studies approved the toxicity of glyphosate on fish as cytogenetic and DNA damage [19,20]. Several studies showed that DNA and chromosomal damage resulted from pollution by Roundup® in blood cells were evaluated by antioxidant responses and oxidation of DNA bases [21]. Single

cell gel electrophoresis (SCGE) assay or Comet assay or microgel electrophoresis (MGE) assay are used to measure DNA damage in single cells. Since the protocol was published by [22]. The Comet Assay is a rapid and quantitative technique. It is based on migration of DNA fragments out of the cell nucleus during electrophoresis. So that, this method has been used in biomonitoring, genotoxicity, ecological monitoring and DNA damage research [23]. There are two types of SCGE techniques. One of them is called neutral Comet assay, where DNA migrates from nucleus as double-stranded DNA breaks (DSB) under neutral condition [24]. The other called alkaline Comet assay, where migration of DNA from nucleus is under alkaline conditions. But this type detects both single-stranded DNA breaks (SSB) and DSB without distinguishing between them [22]. The present study to evaluate the effects of $CdCl_2$, glyphosate and their mixture on DNA damage as biomarkers in the Nile tilapia.

Materials and methods

1-The formula of toxic compounds and their properties:

a.Cadmium Chloride (heavy metal-inorganic compound):

Chemical name: Cadmium Chloride, monohydrate.

M. formula: $CdCl_2.H_2O$ Mol. Wt.: 201.32

Company: LOBA Chemie - India.

b.Glyphosate (herbicide-organic compound):

Chemical name: Glyphosate isopropyl ammonium (Glyphosate IPA).

Trade name: Roundup. Mol. Wt.: 210.

M. formula: $C_6H_{15}N_2O_4P$.

Company: KAFR EL-ZAYAT PESTICIDES & CHEMICALS A. R. E .

2- Determination of lethal concentration LC_{50} for experiments

The freshwater fish Nile tilapia (*Oreochromis niloticus*) fingerlings were collected from a local farm of "Central Laboratory for Aquaculture Research" Abo-Hammad, Sharkia, Egypt. The weight and the length were measured and scored of 32.0±3.0g and 13.0±2.0cm, respectively. Acclimatized fish were distributed in small aquaria to determine the 96h-LC_{50} by Weil equation 1952 [25]. The 96h-LC_{50} were determined for $CdCl_2$ (132mg/l), glyphosate (9.63mg/l), $CdCl_2$ in mixture (41.30mg/l) and glyphosate in mixture (2.75mg/l), respectively were used in the first experiment. Second experiment used 1/4LC_{50} in 8days and 1/10LC_{50} was used in 45days through the third experiment. Three replicates were used for each experiment, as well as control.

3- The physicochemical parameters and some heavy metals of experimental water

The physicochemical parameters of the water used during the experiment were: temperature 24–27°C, pH 7.81–7.89, nitrite 0.081–0.242mg/L, orthophosphate 0.042–0.17mg/L, total ammonia 0.2-0.4mg/L, dissolved oxygen concentration ranged from 7.3 to 7.8mg/L, while total hardness ranged from 190 to 206mg/L and total alkalinity ranged from 75 to 165mg/L as $CaCO_3$. But the concentration of heavy metal in water was measured by atomic absorption apparatus such as Cu, Cd, Zn, Mn, Pb and Fe were 0.027mg/L, 0.00mg/L, 0.111mg/L, 0.083mg/L, 0.00mg/L and 1.775mg/L respectively. Where these results were within permissible limits.

4- Estimation of DNA damage (DNA comet assay)

The Comet assay (CA) or Single-Cell Gel Electrophoresis (SCGE) was developed by [22]. The procedures were described by electrophoresis under pH >13 alkaline conditions as follow: Ten microliter (10ul) from mincing sample of liver or gills mixed with 90ul of Low Melting Point Agarose (LMPA) was added to a fully frosted microscope slide and coated with 110µl of Normal Melting Point Agarose (NMPA). So, cover slip was immediately placed on top of the slide and the agarose layer was allowed to solidify for 10 min at 4ºC. After removal the cover slip the slides were placed in lysis buffer with freshly added 1% Triton (x-100) and 10%DMSO for at least 1hour at 4ºC. Subsequently, slides were placed in the horizontal electrophoresis chamber side by side near one end and close together as possible. Filled the electrophoresis chamber with freshly alkaline buffer until the liquid level completely covers the slides (avoid bubbles over the agarose) for 20min at 4ºC to allow for DNA unwinding and the expression of alkali-labile DNA damage as strand breaks. Electrophoresis was for 30min. at 25V., and 300mA by raising or lowering the running buffer level depending on the purpose on the study and on the extent of migration in control samples and turn off the power after 30min. Gently lift the slides from the buffer and place them on a drain tray. Slides were washed three times with neutralization buffer. Finally, slides were stained with 80ul 1x ethidium bromide, leave for 5min. and then dipped in chilled distilled water to remove excess of stain. The cover slip covers the slides and observed at 400 magnifications in an optica Axioscope fluorescence microscope.

5- Statistical analysis

The experimental data were processed with SPSS 15.0 statistical software. One-way ANOVA (analysis of variance) was performed to estimate the differences

between control and exposure fish to glyphosate, cadmium chloride and mixture. All data were presented as Mean ± S.E. (standard error of the mean). Statistically significant difference was set as $p < 0.05$ [26].

Results and Discussion

The comet assay is one of biological application for detection DNA damage due to the effect of genotoxicants on aquatic organisms. Where, migration of DNA from nucleus expressed as single-strand breaks due to DNA damage as a result of toxicants [27]. Genotoxicology is a tool to describe the effect of toxic substances in aquatic organisms as fish and then man by genotoxicity tests such as DNA damage by using comet assay [28]. Comet assay improved that higher DNA damage in gill cells of fish *Prochilodus lineatus* exposed to formulated glyphosate with Transorb than glyphosate itself [29].

The presented data in table (1) and figures (1, 2, 3) indicate that the effect of 96h-LC_{50} of glyphosate, $CdCl_2$ and their mixture treatment on the DNA damage in gills and liver tissues of *Oreochromis niloticus* after 4 days. Data of comet assay for gills at short time of exposure 4days with high concentration (96h-LC_{50}) showed high DNA damage in mixture treatment followed by glyphosate and $CdCl_2$, respectively. High DNA damage in mixture treatment is due to occurrence of synergistic effect between $CdCl_2$ and glyphosate herbicide also significant increase in %DNA in tail and in tail moment. High DNA damage caused by glyphosate is due to emulsifier surfactant POEA. But moderate DNA damage of $CdCl_2$ despite of its high toxicity is due to short time of exposure. Data of comet assay for liver at short time of exposure 4days with high concentration (96h-LC_{50}) showed high DNA damage in $CdCl_2$ treatment followed by mixture and glyphosate, respectively. This research suggested that high DNA damage of $CdCl_2$ in liver cells is due to the liver as a store of heavy metals where cadmium is non-essential element also high %DNA and high tail moment. But, DNA damage of mixture treatment is quite high caused by occurrence weak synergistic effect between $CdCl_2$ and glyphosate. Moderate DNA damage of glyphosate treatment and high significant increase of %DNA in tail and tail moment is due to formulation of glyphosate. These results agree with most previous studies as significant increase in tail length, tail DNA% and T.M. in organophosphate insecticides fenitrothion (FNT) on gill tissues of Nile tilapia, *Oreochromis niloticus* via exposure period 96h-LC_{50} [30]. DNA damage improved by comet assay after exposure period 3, 14days by analysis of hepatic cells of

Anguilla Anguilla fish exposed to Roundup® [31]. The comet assay of gill cells scored significantly higher DNA damage of *P. lineatus* erythrocytes after 96h. of exposure to Roundup® [20]. Liver and gill cells of *Anguilla anguilla* fish showed increasing in DNA damage for the two Roundup® concentrations (58 and 116µg/L) after exposure period of 3days [32]. Also, comet assay showed higher DNA damage occurred for exposed liver, kidney and gill cells of *Oreochromis niloticus* to di-n-butyl phthalate (DBP) for 4days of 1/2 (96h. LC_{50}) than 1/3(96h. LC_{50}) [33]. Also, high DNA damage was observed by comet assay of gills of *Labeo rohita* exposed to 33.6, 67.1 and 100.6mg/L of cadmium chloride at 96h. [34]. The data presented in table (2) and figures (4,5) indicate that the effect of $1/4LC_{50}$ of glyphosate, $CdCl_2$ and their mixture treatment on the DNA damage markers in gills and liver of *Oreochromis niloticus* after 8 days. Data of comet assay for gills at moderate time of exposure 8 days with quite high concentration of $1/4LC_{50}$ showed high DNA damage in $CdCl_2$ treatment followed by glyphosate and mixture, respectively. High DNA damage of $CdCl_2$ is due to its high toxicity also high %DNA in tail and tail moment. Moderate DNA damage of glyphosate is because of its composition also moderate significance of %DNA in tail and tail moment. Low DNA damage of mixture treatment is due to occurrence antagonistic effect between $CdCl_2$ and glyphosate herbicide also significant decrease in %DNA in tail and tail moment. Data of comet assay for liver at moderate time of exposure 8 days with quite high concentration of $1/4LC_{50}$ showed that quite high DNA damage in all treatments where it is thought that DNA damage reached at this level of damage and stopped or got a repair system. But toxicity of these pollutants causes significant increasing in %DNA in tail and tail moment. These results are similar to the results of the exposure of *Anguilla Anguilla* fish to the herbicide commercial formulations Roundup® and Garlon® formulations for 3, 7 and 14days.Where, it shows high DNA damage [35]. Also, the experimental field proved that pollution of River Nile with heavy metals as cadmium led to significant increasing of DNA tail length, percentage of DNA and tail moment in blood cells of *Oreochromis niloticus* collected from polluted area compared to that of reference by comet assay [36]. Comet assay proved that high DNA damage of blood cells of Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) in field study on river Nile of Egypt in polluted regions with heavy metals specially cadmium [37]. Also, comet assay proved that significant

increasing of DNA damage of blood cells of Nile tilapia (*Oreochromis niloticus*) in field study on Guaribas river of Brazil in polluted regions with heavy metals such as Fe, Zn, Cr, Cu and Al. [38]. The data presented in table (3) and figures (6, 7) indicate that the effect of $1/10LC_{50}$ of glyphosate, $CdCl_2$ and their mixture treatment on the DNA damage markers in gills and liver of *Oreochromis niloticus* after 45days. Data of comet assay for gills at long time of exposure 45 days with low concentration of $1/10LC_{50}$ showed high DNA damage in mixture treatment followed by $CdCl_2$ and glyphosate, respectively. High DNA damage in mixture treatment is because of occurrence synergistic effect between $CdCl_2$ and glyphosate herbicide also significant increasing in %DNA in tail and in tail moment. Quite high DNA damage of $CdCl_2$ is because of its being potent toxic element also high %DNA in tail and tail moment. Also, limited high DNA damage of glyphosate due to its composition and high significance of %DNA in tail and tail moment. Data of comet assay for liver after long time of exposure 45 days with low concentration $1/10LC_{50}$ showed high DNA damage in mixture treatment followed by $CdCl_2$ and glyphosate, respectively. This research suggested that high DNA damage of mixture treatment is due to occurrence synergistic effect between $CdCl_2$ and glyphosate. High DNA damage of $CdCl_2$ treatment due to presence of sufficient amount of cadmium in liver that led to high significance of %DNA in tail and tail moment. Limited high DNA damage in glyphosate treatment due to the toxic compounds which is added to glyphosate during its commercial preparation also quite high significance of %DNA in tail and tail moment. These results obtained from experiment of long exposure period to pollutants were similar to [39]. where, exposure to low levels of Cd can cause stress on (*Cyprinus carpio*) led to DNA damage. The comet bioassay illustrated DNA damage and oxidative stress resulted from the effect of metallic ion concentrations (Cd and Pb) and exposure period (30days) led to %DNA damage, tail length and tail moment were significantly increased in common carp [40]. The comet assay showed the same results obtained by [41]. Also, DNA damage occurred for exposed liver cells of *Oreochromis niloticus* to diclofenac (DCF) for 60days showed as prolonged exposure period [42].

Conclusion:

From that results it can be concluded that the effects of $CdCl_2$, glyphosate and their mixture on DNA damage in Nile tilapia caused DNA damage because of the toxicity of $CdCl_2$ and glyphosate. High DNA damage for mixture was caused by

occurrence synergistic effect between $CdCl_2$ and glyphosate. So, DNA damage as molecular biomarkers in the Nile tilapia of pollution.

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Table 1: Mean ± SE of %DNA damage (comet assay) observed in gills and liver of Nile tilapia fingerlings after exposure period 4days.

Tissues	Exposure period (96hrs.) 4days with LC_{50}			
	Treatments	Comet assay (DNA damage) Mean ± S. E		
		Tail length	%DNA in tail	Tail moment
Gills	Control	$3.49\pm0.21^{b,d}$	$11.56\pm0.58^{b,c,d}$	$0.44\pm0.01^{b,c,d}$
	Glyphosate	4.42 ± 0.13^{a}	$21.23\pm1.82^{a,d}$	$1.13\pm0.11^{a,d}$
	Cadmium	3.86 ± 0.22^{d}	$22.57\pm0.48^{a,d}$	$1.04\pm0.03^{a,d}$
	Mixture	$4.86\pm0.39^{a,c}$	$27.09\pm0.89^{a,b,c}$	$1.56\pm0.22^{a,b,c}$
Liver	control	3.12 ± 0.07^{c}	$9.60\pm0.54^{b,d}$	$0.31\pm0.02^{b,c,d}$
	Glyphosate	4.21 ± 0.33	$23.98\pm1.66^{a,c,d}$	0.94 ± 0.06^{a}
	Cadmium	6.88 ± 2.01^{a}	$12.20\pm1.31^{b,d}$	0.86 ± 0.28^{a}
	Mixture	4.30 ± 0.17	$19.53\pm1.19^{a,b,c}$	0.97 ± 0.15^{a}

(a, b, c, d: significance comparing with control, glyphosate, cadmium and mixture, respectively).

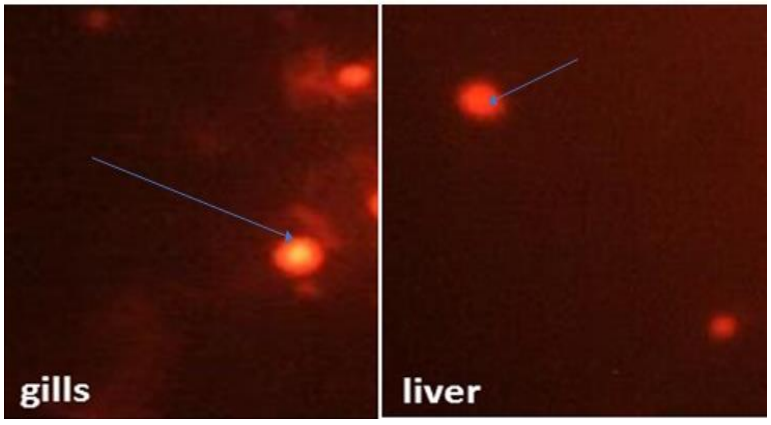

Figure 1. DNA profile of control group in gills and liver of Nile tilapia by comet assay.

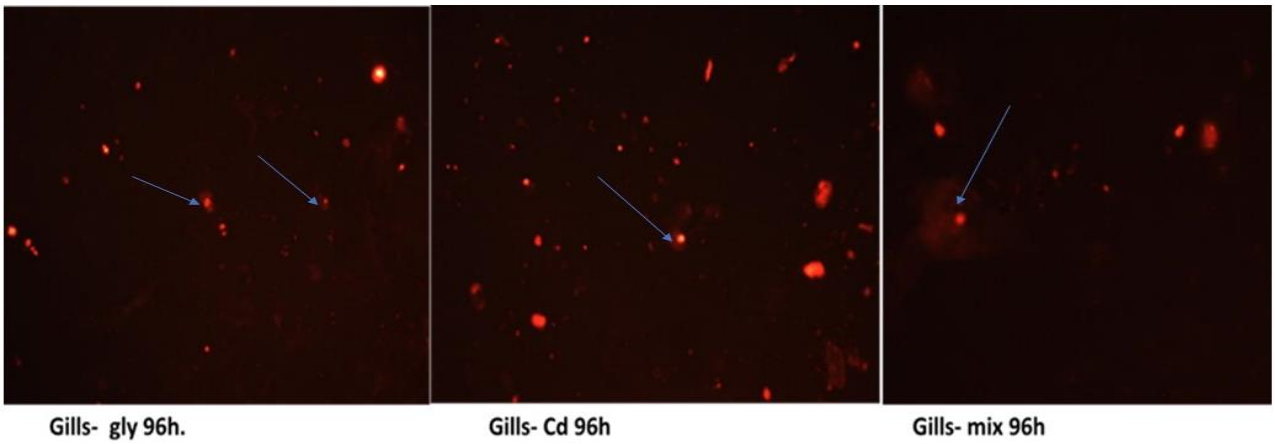

Figure 2. DNA damage profile of gills tissues after exposure to 96h. glyphosate, $CdCl_2$ and mixture in Nile tilapia by comet assay.

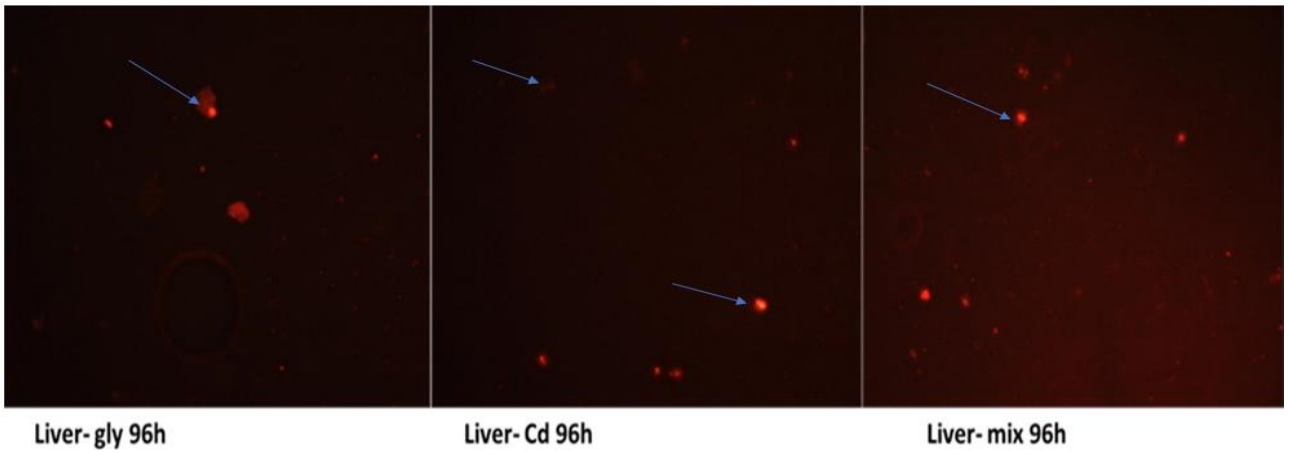

Figure 3. DNA damage profile of liver tissues after exposure to 96h. glyphosate, $CdCl_2$ and mixture in Nile tilapia by comet assay.

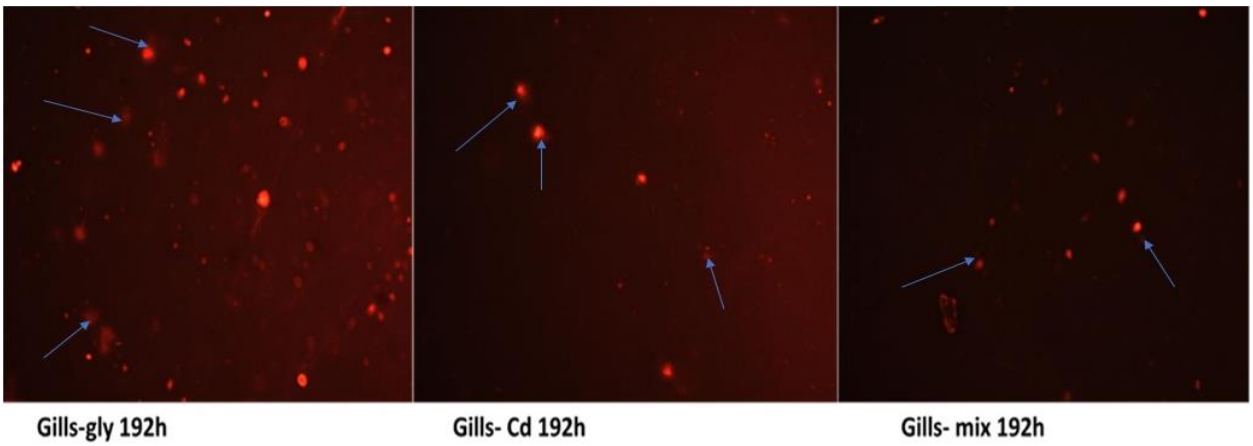

Figure 4. DNA damage profile of gills tissues after exposure to 192h. glyphosate, $CdCl_2$ and mixture in Nile tilapia fingerlings by comet assay.

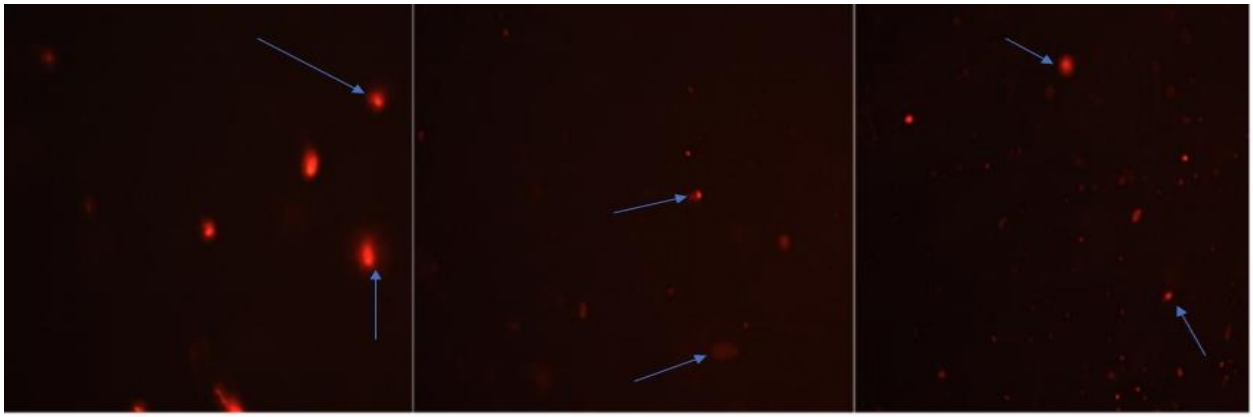

Figure 5. DNA damage profile of liver tissues after exposure to 192h. glyphosate, $CdCl_2$ and mixture in Nile tilapia fingerlings by comet assay.

Table 2: Mean ± SE of %DNA damage (comet assay) observed in gills and liver of Nile tilapia fingerlings after exposure period 8days.

Tissues	Exposure period (192hrs.) 8 days with 1/4 LC_{50}			
	Treatments	Comet assay (DNA damage) Mean ± S. E		
		Tail length	%DNA in tail	Tail moment
Gills	control	$3.48\pm0.21^{b,c,d}$	$11.55\pm0.58^{b,c,d}$	$0.43\pm0.01^{b,c}$
	Glyphosate	$4.07\pm0.12^{a,c,d}$	21.17 ± 1.77^{a}	$1.06\pm0.09^{a,c,d}$
	Cadmium	$5.12\pm0.17^{a,b,d}$	20.34 ± 2.07^{a}	$1.33\pm0.05^{a,b,d}$
	Mixture	$2.19\pm0.09^{a,b,c}$	19.21 ± 0.47^{a}	$0.48\pm0.04^{b,c}$
Liver	control	3.12 ± 0.07	9.60 ± 0.54^{b}	$0.31\pm0.02^{b,d}$
	Glyphosate	4.79 ± 0.59	19.30 ± 4.85^{a}	$1.23\pm0.13^{a,c}$
	Cadmium	4.30 ± 0.59	15.17 ± 0.84	0.62 ± 0.05^{b}
	Mixture	4.70 ± 0.95	13.50 ± 2.81	0.84 ± 0.26^{a}

(a, b, c, d: significance comparing with control, glyphosate, cadmium and mixture, respectively).

Table 3 – Mean ± SE of %DNA damage (comet assay) observed in gills and liver of Nile tilapia fingerlings after exposure period 45days.

Tissues	Exposure period 45days with 1/10LC_{50}			
	Treatments	Comet assay (DNA damage) Mean ± S. E		
		Tail length	%DNA in tail	Tail moment
Gills	control	3.48±0.21^{b,c,d}	11.55±0.58^{b,c,d}	0.44±0.01^{b,c,d}
	Glyphosate	4.51±0.29^{a,d}	22.09±0.91^{a}	1.20±0.13^{a}
	Cadmium	4.634±0.194^{a}	23.90±1.55^{a}	1.23±0.09^{a}
	Mixture	5.32±0.24^{a,b}	20.57±1.66^{a}	1.26±0.15^{a}
Liver	Control	3.12±0.07^{b,c,d}	9.61±0.54^{b,c,d}	0.31±0.02^{b,c,d}
	Glyphosate	4.24±0.14^{a}	15.15±0.39^{a,c,d}	0.78±0.02^{a,c,d}
	Cadmium	4.60±0.35^{a}	20.27±0.39^{a,b}	1.06±0.14^{a,b,d}
	Mixture	4.60±0.35^{a}	20.27±0.39^{a,b}	1.29±0.04^{a,b,c}

(a, b, c, d: significance comparing with control, glyphosate, cadmium and mixture, respectively)

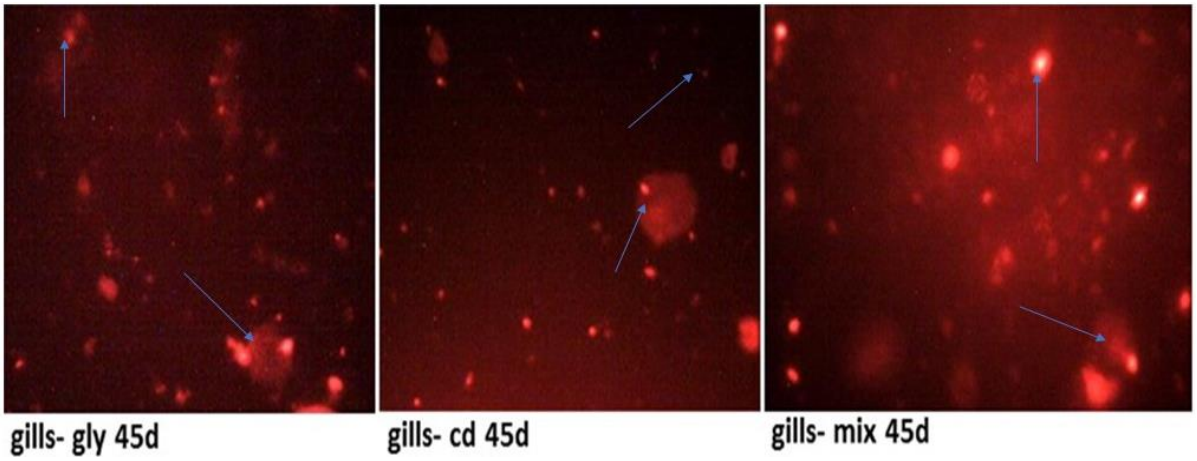

Figure 6. DNA damage profile of gills tissues after exposure to 45days glyphosate, $CdCl_2$ and mixture in Nile tilapia fingerlings by comet assay.

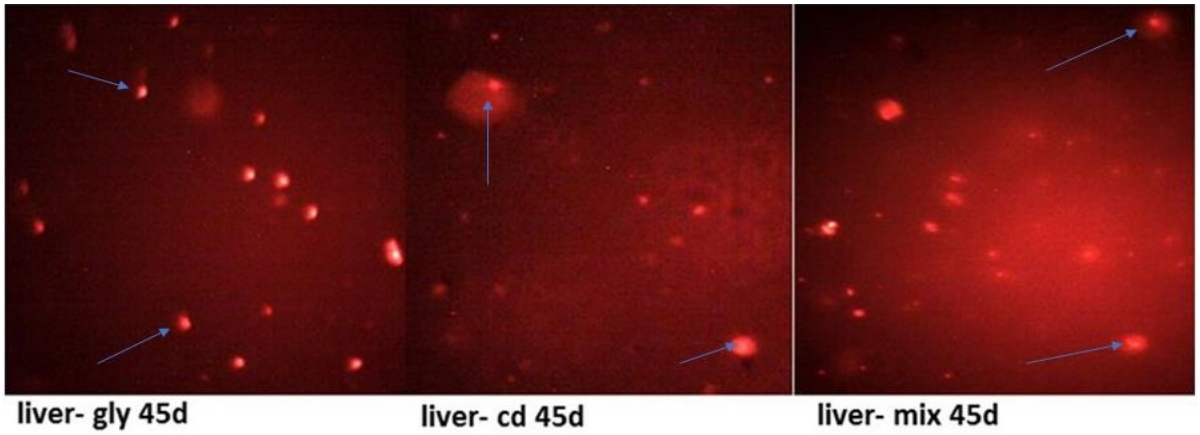

Figure 7. DNA damage profile of liver tissues after exposure to 45days glyphosate, $CdCl_2$ and mixture in Nile tilapia fingerlings by comet assay.