Studying of genotoxicity of glyphosate herbicide on mammal cells

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Abstract

Glyphosate herbicide used to control unwanted annual and perennial plants. Roundup, one of the most widely used products containing glyphosate, is classified as hazardous to the environment. Therefore, this study aim to assess the effect of (25mg/kg) and (50mg/kg) of glyphosate, given to mice by "gavage" and "feeding", by exposure of *Cyprinus carpio* (Common carp) to the herbicide with the same concentration and then given to mice, and noticeable cytogenetic effects (DNA damage) on mice bone marrow cells (*in vivo*) by using Comet assay. The statistical analysis shows that glyphosate cause significant increase ($P \leq 0.01$) of DNA damage of bone marrow cells in mice in comparing with the negative controls.

Introduction

Herbicides are chemicals used to manipulate or control undesirable vegetation. The most frequent application of herbicides occurs in row-crop farming, where they are applied before or during planting to maximize crop productivity by minimizing other vegetation [1]. Glyphosate (N-phosphonomethyl-glycine) is a post-emergence herbicide used for weed control in various crops, especially rice, maize and soybean [2]. The commonly accepted explanation of glyphosate's mode of action is as follows: glyphosate inhibits the enzyme 5-enolpyruvyl-shikimate 3-phosphate synthase (EPSPS), which is essential for the formation of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) in plants, by what is commonly referred to as the shikimic pathway [3]. Roundup, one of the most widely used products containing glyphosate, is classified as hazardous to the environment. It was launched onto the American market in 1990 for the control of weeds in sugar cane, coffee, and citrus plantations. In comparison with other formulations, the main characteristic of this product is its rapid absorption, aided by the presence of surfactants. Roundup contains a mixture of 15% polyoxyethylene amine (POEA) with

other unspecified surfactants [4]. Previous studies have reported that this formulation is more toxic than glyphosate alone [5].

Glyphosate is a polar, highly water-soluble substance that easily forms complexes with metals and binds tightly to soil components [6], but can be desorbed or leached out of the soil, thus, traces of this compound have been found in surface-water and groundwater systems [7]. There are many studies demonstrated that the glyphosate, Roundup, and/or the metabolite to be genotoxic. Even some industry papers show this. Roundup caused dose-dependent DNA damage in human liver cells, with 50% DNA strand breaks at 5 mg/kg [8]. Glyphosate caused the induction of micronuclei at high doses, possibly through oxidative stress, in mice bone marrow [9]. The Comet assay (also known as single cell gel electrophoresis) is a straightforward and highly sensitive method for measuring DNA damage and repair at the level of individual cells [10]. The idea was to combine DNA gel electrophoresis with fluorescence microscopy to visualize migration of DNA strands from individual agarose-embedded cells. If the negatively charged DNA contained breaks, DNA supercoils were relaxed and broken ends were able to migrate toward the anode during a brief electrophoresis. If the DNA was undamaged, the lack of free ends and large size of the fragments prevented migration. Determination of the relative amount of DNA that migrated provided a simple way to measure the number of DNA breaks in an individual cell [11].

Materials and Methods

Laboratory Animals

Albino Swiss male mice (*Mus musculs*) were used in the experiments. their ages ranged between 8-12 weeks with a body weight ranged between 25-30 g. Mice were obtained from the colony of the animal house of the Al-Nahrain University, Institute of Embryo Research and Infertility Treatment, and National center for drug control and researches. They were kept in a room supplied with air conditioner to keep the temperature between18-24 ˚C, the air of the room was changed continuously by using ventilating fan and light was controlled with range of 12 hours of light and 12 hours of darkness. The animals were housed in plastic cages (2mice\cage) with a wire grid covers, supported on ventilated racks [12].

The used herbicide

The used herbicide was glyphosate in the form of commercial formulation (Roundup) manufactured by Monsanto Europe N.V Belgium with concentration of 360g/l that obtained from Baghdad markets (specialized with herbicides control). The herbicide was diluted into (25

and 50) mg/kg b. wt. of mice and gave by administration and to common carp by exposure.

Animals Groups

The experiment was achieved as following:

1. All the animals were divided into two groups:
   1st group: were administered the diluted glyphosate herbicide (25 and 50mg/kg).), while the corresponding control animals received distilled water only
   2nd group were given *cyprinus carpio* that treated by the same concentrations of glyphosate herbicide. While the control group was given untreated carp.
2. All these groups were treated seven days before week from examination.

Methods of Comet assay

Thirty six mice male and female were used in the experiment (18male and 18 female); the animals were divided into two groups (show above).

The animals were sacrificed by cervical dislocation and the thigh bone was taken and cleaned from tissue and muscles, then catched from the middle with a forceps in vertical position over the edge of test tube. By a sterile syringes, (5 ml) of warm Phosphate buffer saline (PBS) 37ºC was injected as to wash and drop the bone marrow in the test tube to formation of cell suspension.

The comet assay was performed under alkaline condition. Essentially according to [13] with slight modification.

Agarose preparation

- 1% low-gelling-temperature agarose was prepared by mixing powdered agarose (0.5) with distilled water (50) in a glass beaker or bottle.
- Bottle was placed in the 100 °C water bath for several minutes. (Avoided boiling of the agarose and ensure that all agarose is dissolved).
- Bottle was placed with agarose into a 40 °C water bath.

Preparation of Samples and Slides

- Agarose slides were prepared by dipping the slides into normal molten 1% (w/v) agarose.
- Agarose was allowed to air-dry to a thin film. Slides can be prepared ahead of time and stored with desiccant.
- Cells suspension was centrifuged at 1500 rpm for 2 min. The supernatant was discarded and the pellet washed once with ice-cold PBS

(without Mg^{2+} and Ca^{2+}) and centrifuged at 1500 rpm for 2 min. then supernatant was discarded.

- Cell sample was combined with low melting point agarose at 0.5 (w/v) and the mixture (75µl/ well) immediately was added into slide comet by pipette.
- The slides were hold horizontally then transferred to 4ºC in a dark container for 30 min.
- The slide was transferred to a small basin overnight (18−20 h) at 4 °C in the dark.
- After overnight, the slide was immersed with electrophoresis buffer solution for 20 min.
- The slides were hold horizontally, then transferred to a horizontal electrophoresis chamber filled with a cold electrophoresis solution, 24volt and 300mA was applied to the chamber for 18 min.
- TBE electrophoresis solution was aspirated from chamber and replaced with 0.4 M of Tris-HCl solution (pH 7.5) for 5 min in order to neutralize of cells (The step repeated twice).
- Diluted ethidium bromide dye 50 µl was added to each well comet assay slide and incubated at room temperature for 10 min.
- The slides were rinsed with distilled water to remove excess stain.
- The slides were examined by fluorescence microscope.

Results

The results of the comet assay, summarized in the table (1) give the mean values of tail length, % DNA mean in tail and tail moment and olive tail moment for experimental animals and control groups. The mean of tail length (Mean ± SE) of control group and animals treated with herbicide normal and double dose (gavage and feeding) are 2.8±0.08px, 6.8±0.285px, 8.9±0.244px, 3.71±0.408px, 4.9±0.244px, 6.3±0.285px, respectively (px. Pixel is length unit; one pixel is equal to one dot on the computer). According to the results obtained here, the experimental animals are highly significant ($P \leq 0.01$) compared with control groups table (1). It is found in the comet assay that the % DNA mean in tail (Mean ±SE)) of control group and animals treated with herbicide normal and double dose (gavage and feeding) are 1.09±0.012, 7.23±0.408, 9.62±0.408, 4.83±0.326, 5.77±0.379, 7.09±0.412, respectively. There is a significant increase between experimental animals and controls for % DNA Mean in tail ($P \leq 0.01$). DNA damage is statistically significantly ($P \leq 0.01$) in case of experimental animals for tail moment (Mean ±SE) are 0.22±0.001, 0.43±0.028, 0.13±0.01, 0.2±0.01 respectively than in control subjects 0.02±0.0009, 0.08±0.004. The mean of olive tail moment (Mean ± SE) of control group and animals treated with herbicide normal and double dose

(gavage and feeding) are 0.06±0.016, 0.44±0.024, 0.67±0.032, 0.12±0.004, 0.22±0.004, 0.46±0.016 respectively. There is a significant increase between experimental animals and controls for Olive tail moment (P ≤0.01). As shown in table (1) (figure 1).

Table (4-2): The results of comet assay (Mean ± SE) for bone marrow of mice treated with glyphosate herbicide (gavage and feeding) and control groups as mean value of the measurements of 100 comets per subject.

Treatment	Tail length	Tail DNA %	Tail moment	Olive Tail Moment
Control	2.8±0.08^{e}	1.09±0.012^{d}	0.02±0.0009^{c}	0.06±0.016^{e}
Normal dose (gavage)	6.8±0.285^{b}	7.23±0.408^{b}	0.22±0.001^{c}	0.44±0.024^{b}
Double dose (gavage)	8.9±0.244^{a}	9.62±0.408^{a}	0.43±0.028^{a}	0.67±0.032^{a}
Control (feeding)	3.71±0.408^{d}	4.83±0.326^{c}	0.08±0.004^{d}	0.12±0.004^{d}
Normal dose (feeding)	4.9±0.244^{c}	5.77±0.379^{c}	0.13±0.01^{c}	0.22±0.004^{c}
Double dose (feeding)	6.3±0.285^{b}	7.09±0.412^{b}	0.2±0.01^{b}	0.46±0.016^{b}

The different letters between treatments denoted that significant differences at level (P≤0.01).

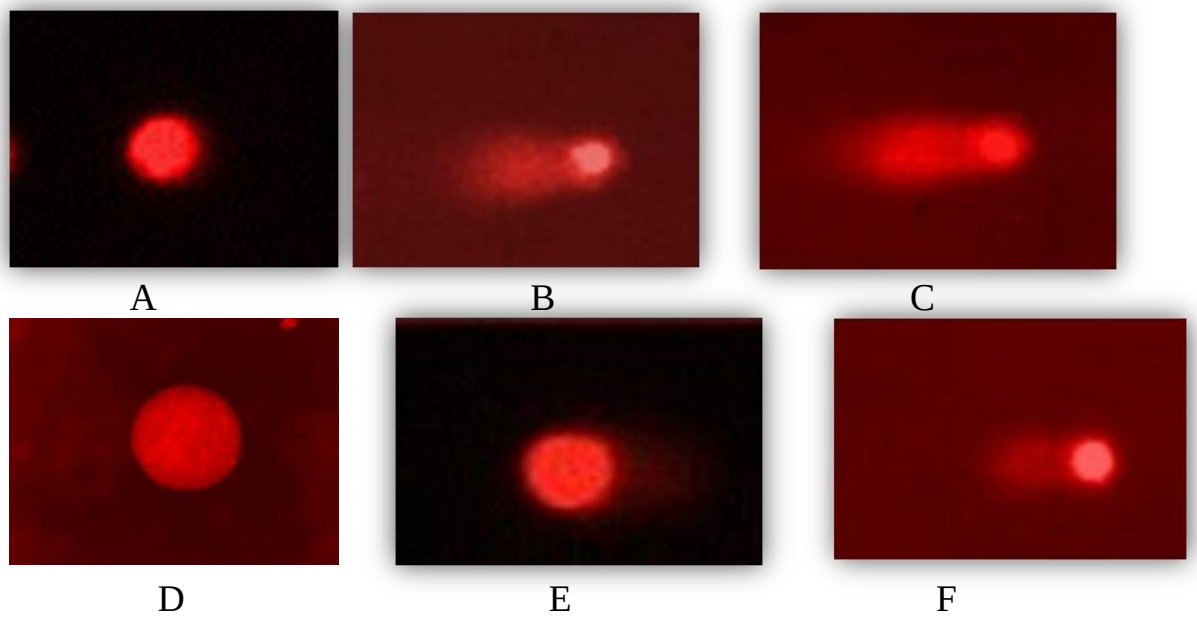

Figure (1): Comet images by florescent microscope (400X)
(A) Undamaged DNA (control) (B) Damaged DNA (treated with normal dose of glyposate) (C) Damaged DNA (treated with double dose of glyphosate) (D) Undamaged DNA (control "feeding") (E)Damaged DNA (treated with normal dose of glyphosate by feeding) (F) Damaged DNA (treated with double dose of glyphosate by feeding).

Discussion

The genotoxicity of glyphosate and Roundup *in vivo* recorded cytogenic damage in mouse bone marrow which was more pronounced for Roundup. A DNA-damaging activity of glyphosate and Roundup was also observed in the mice's liver and kidneys [14] .The toxicity and genotoxicity evaluation carried out more than 10 years ago classified glyphosate as a low-risk herbicide for animal and human health. Conclude their review by stating that "under the conditions of present and expected use, there is no possibility that glyphosate poses a risk to human health" [15]. This review disagreed by [9] who proved The comet assay provided evidence that glyphosate produces DNA damage both *in vitro* and *in vivo*. A series of tests was conducted to determine the genotoxic potential of glyphosate and its main degradation product, AMPA. A statistically significant increase in levels of chromosome aberrations with a concentration of 200 mg/ml of AMPA was found by the CA test in human peripheral blood cells.

Moreover, the comet assay showed evidence of DNA damage in different human cell lines for glyphosate and AMPA [16] and in liver and kidney of mice intraperitoneally injected with 300 mg/kg of glyphosate [17]. Other some studies showed a statistically significant increase in the index of DNA damage was obtained in peripheral blood from Balb C mice exposed to 400 mg/kg of glyphosate in the same way [18].

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دراسة السمية الوراثية لمبيد الكلايفوسيت على خلايا اللبائن

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المستخلص

يستخدم مبيد الكلايفوسيت للسيطرة على النباتات الحولية والمعمرة الغير مرغوب فيها. يعد الراوند اب احد المنتجات المشتقة من الكلايفوسيت الأكثر استخداما على نطاق واسع ، وتصنف على أنها خطرة على البيئة. لذلك فإن هذه الدراسة تهدف إلى تقييم تأثير مبيد الكلايفوسيت باستخدام تركيزين للمبيد (25 , 50) ملغم/ كغم , اعطيت هذه التراكيز للفئران بواسطة التجريع الفموي وكذلك عن طريق التغذية بأسماك الكارب المعرضة مسبقاً للمبيد لنفس التراكيز المذكورة وملاحظة التأثيرات الوراثية (تلف الحامض النووي) على خلايا نقي العظم للفئران (داخل الجسم) وذلك باستخدام تقنية المذنب القاعدي. اظهرت النتائج الاحصائية ان مبيد الكلايفوسيت يسبب زيادة معنوية ($P\leq0.01$) لتلف الحامض النووي في خلايا نقي العظم للفئران بالمقارنة مع السيطرات السالبة.