Biohelikon: Cell Biology(ISSN: 2348-3741), 2014 2:a14

Research

Orange juice reduced genetic and biochemical alterations induced by herbicide Glyphosate in mice

Mahrousa M.Hassanane[1*], Salwa M.Kassem[1], Safinaz E. Eltoukhy[2]

[1]Cell Biology Department, National Research Center, Egypt.

[2]Medical Biochemistry Department, National Research Center, Egypt.

[*] Corresponding author: Dr. Mahrousa M.Hassanane, Email: mahrousamh@yahoo.com

Abstract

Glyphosate (N-(phosphonomethyl) glycine, C_3 H_8 NO_5P) is the world's most widely used herbicide. Nevertheless their benefits, occupational and environmental exposure to pesticides can pose a threat to non-target species including human beings. Numerous laboratory studies have shown that Glyphosate and the roundup formulation can be genotoxic and endocrine disrupting. Therefore, the present study was undertaken to evaluate the genotoxic and biochemical effects of Glyphosate as well as the protective effects of orange juice against these alterations in Swiss albino male mice. Animals were administered orally with the pesticide and/or orange juice for a period of 2 and 4 weeks and divided into 4 groups (10 animals each) as follows: The 1st group served as control, the 2nd group was treated with orange juice, the 3rd group was treated with pesticide Glyphosate and the 4th group was treated with pesticide in combination with orange Juice. The results revealed that treatment with Glyphosate induced DNA damage, micronucleus formation and chromosomal aberrations in bone marrow and spermatocyte cells, as well DNA fragmentation potentially by increasing generation of free radicals which are reconvened by orange juice treatment as a protective agent. The biochemical parameters (malondialdehyde levels, MDA) were increased significantly in the Glyphosate treated groups, whereas the combination with orange juice reduced that elevation in these parameters. In conclusion, Glyphosate appears to have the ability to cause genetic damage and biochemical alterations in the treated mice. The protective role of orange juice could be attributed to its antioxidant properties.

Keywords

Glyphosate, Genotoxic, Biochemical Alterations, Protective, Orange Juice, Mice

Biohelikon

Introduction

Pesticides, including herbicides, insecticides, and fungicides are used extensively to improve crop yields and as a result, they accumulate in the environment and humans get unavoidably exposed to them [1]. Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA [2,3]. Because of their biological activity, the indiscriminate use of pesticides may cause undesired effects to human health. For instance, the induction of DNA damage can potentially lead to adverse reproductive outcomes, carcinogenesis, and many other chronic diseases [4-7]. Epidemiological studies demonstrated that occupational exposure to some pesticides may be related to several kinds of cancer, including leukemia [8], bladder [9], and pancreatic cancers [10].

Glyphosate (N-(phosphonomethyl) glycine, C_3 H_8 NO_5P), commonly known by its original trade name Roundup™ (manufactured by Monsanto), is the world's most widely used herbicide. Glyphosate-based herbicides are manufactured by many companies in many countries. Some researchers have concluded that Glyphosate and its formulations clearly present a risk of carcinogenic, mutagenic, and reproductive effects on human cells [11].

Numerous laboratory studies have shown that Glyphosate and the Roundup formulation can be genotoxic and endocrine disrupting. One study summarizes these effects occurring at doses substantially lower than those used in agriculture, or permitted as residues: at 0.5 mg/kg (40 times lower than levels permitted in soybeans in the US). They were anti-androgenic; at 2 mg/kg they were anti-oestrogenic; at 1 mg/kg they disrupted the enzyme aromatase; at 5 mg/kg they damaged DNA, and at 10 mg/kg they were cytotoxic. These effects can result in crucial outcomes for sexual and other cell differentiation, bone metabolism, liver metabolism, reproduction, development and behavior, and hormone dependent diseases such as breast and prostate cancer [12].

A variety of tests on animals, bacteria, and plant cells have further demonstrated the genotoxic ability of Glyphosate, where the Glyphosate caused the induction of micronuclei at high doses, possibly through oxidative stress, in mouse bone marrow [13].Glyphosate caused chromosomal aberrations and sister chromatid exchange in bovine lymphocytes *in vitro* [14]including at concentrations of 56 to 1120 μM [15]. Glyphosate was found to increase malondialdehyde (MDA) levels as reported by Cavuşoğlu et al. [16].

Oranges are among the most consumed fruits worldwide, which reflects the fact they have been cultivated since ancient times. They are widely grown in warm climates worldwide, with Brazil and the United States producing about 50% of the total world supply. The flavors of oranges vary from sweet to sour. The fruits commonly peeled and eaten fresh or squeezed for its juice, which can vary from sweet to sour. There are 2 kinds of orange; *Citrussinensis* that is called sweet orangeand *Citrus aurantium,* whichis called sour orange (other names include bitter orangebigarade orange and Seville orange) [17]. Sweet oranges can be further divided into 2 varieties, blond and blood oranges, based in their pulp coloration. Blond oranges have a smooth peel, soft pulp, weakly winged leafstalk [18] and regular-colored juice. Blood (red pulp) oranges are richer inanthocyanins and produce darker juices [19].

Orange juice is among the most consumed fruit juices and is considered among the top micronutrient-dense juices (i.e., a food that provides substantial amounts of vitamins and minerals and relatively fewer calories) [20]. Orange juice itself and its constituents may exert various biological effects [21-23].

Recent evidence from a study comparing adults over 19 years of age (n =8,861), who were consumers and non consumers of orange juice participating in the National Health and Nutrition Examination Survey, 2003–2006 have shown that consumption of orange juice (usual per capita intake of 100% orange juice was 50.3 mL/day) was associated with better diet quality and an increased prevalence of meeting the estimated average requirement for key nutrients (mainly vitamin C and folate). The study also indicated that consumers of orange juice had higher prevalence of other biomarkers of positive health outcomes, including lower total cholesterol and LDL levels, and had lower mean body mass index and a decreased risk of obesity [24].

Therefore, the present study was undertaken to evaluate the genotoxic and biochemical effects of Glyphosate as well the protective effects of orange juice against these alterations on Swiss albino male mice.

Materials and Methods:

Chemicals

Pesticide Glyphosate (N-(phosphonomethyl)glycine), commonly known by its original trade name Roundup™ (manufactured by Monsanto), was obtained from Sigma Chemical Company-St Louis, MO, USA. Orange Juice was supplied by Juhayna and beverages company, Giza, Egypt.

Animals and treatment

Adult Swiss albino male mice weighting about 252 gm, obtained from the animal house colony of the National Research Center, Dokki, Cairo, Egypt, were used in the study as males have been shown to be more susceptible than females to genotoxic effects of various chemicals. The animals were housed in polypropylene cages, given water *ad libitum* and fed standard pellets diet for I week for adaptation. Mice were exposed to a 12:12 light/ dark cycle, at a room temperature of 18-22°C, and were administered orally pesticide and / or orange juice for a period of 2 and 4 weeks and divided into 4 groups: 10 animals each as follow: 1-The 1st group treated orally with distilled water and served as control. 2- The 2nd group treated with orange juice (10 ml/kg) daily. 3- The 3rd group treated with pesticide. 4-The 4th group treated with pesticide in combination with orange Juice.

Collection of blood samples:

Mice were anaesthetized by diethyl ether and samples were taken from retro orbital plexus using glass capillaries. Blood samples were collected at the end of the 2nd and the 4th week of treatment for determining DNA damage using the comet assay.

Comet assay

Isolated blood cells of all groups of male mice were subjected to the modified single-cell gel electrophoresis or comet assay [25]. To obtain the cells, the pellet of blood cells was washed with an excess of ice-cold Hank's balanced salt solution (HBSS) and minced quickly into approximately 1 mm^3 pieces while immersed in HBSS, with a pair of stainless steel scissors. After severalwashings with cold phosphate-buffered saline (to remove red blood cells), the blood cells were dispersed into single cells using a pipette. In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold). The cells were treated with alkali for 20 min to denature the DNA and subjected to a 30 min electrophoresis under alkaline conditions at 300 mA, 25 V. The slides were stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX60 F-3) with a green filter at 40X magnification lens (N.A.=1.3). For each experimental condition, about 100 cells (about 25 cells per animal) were examined to determine the percentage of cells with DNA damage that appear like comets. The non-overlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between 1× and 2× the nuclear diameter; and class 3 = tail longer than 2× the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus [26]. A total damage score for each slide was derived by multiplying the number of cells assigned to each class of damage by the numeric value of the class and summing up the values. Slides were analyzed by one observer to minimize the scoring variability.

Micronucleus test:

Bone marrow slides were prepared according to the method described by Adler[27]. The bone marrow was washed with 1 ml of fetal calf serum and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 minutes, followed

by staining in May-Grunwald Giemsa for 5 minutes then washed in distilled water and mounted. For each animal, 2000 polychromatic erythrocytes (PCEs) were examined for the presence of micronucleated polychromatic erythrocytes (%MnPCEs). Two hours before sacrifice, the animals were injected with 0.5 mg of colchicines solution which completely inhibits the cell division (*mitosis*).

Cytogenetic analysis (chromosomal aberrations)

Mice were subjected to cytogenetic analysis from bone marrow cells using the method of Preston et al., [28]. Briefly, mice were injected intraperitoneally (i.p) with colchicines (0.05 mg/kg) for two and a half hours before sacrifice. Animals were sacrificed and femoral bone marrow cells were flushed with isotonic solution (0.9% NaCl). Hypotonic solution (0.56% KCl) was added to the cell pellet and incubated at 37°C for 30 min. the solution was fixed, slides were prepared and stained using 10% Giemsa stain. Fifty metaphase spreads for animals were analyzed for scoring the different types of chromosomal aberrations. The results were statistically analyzed using chi-square test.

Also, spermatocyte cells were prepared for meiotic chromosomal analysis according to Brewen and Preston [29].

DNA fragmentation:

Liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml lysis buffer containing 10mM tris-HCL (pH.8), 1 mM EDTA, 0.2 % Triton X-100, centrifuged at 10000 *g* (Eppendorf) for 20 minutes at 4°C. The pellets were re-suspended in 0.5 ml of lysis buffer. To the pellets (P) and supernatants (S), 1.5 ml of 10 % trichloroacetic acid (TCA) was added and incubated at 4°C for 10 minutes. The samples were centrifuged for 20 minutes at 11952 *g* (Eppendorf) at 4°C and the pellets were suspended in 750 µl of 5 % TCA, followed by incubation at 100°C for 20 minutes. Subsequently, to each sample 2 ml of DPA solution [200 mg DPA in 10 ml glacial acetic acid, 150 µl of sulfuric acid and 60 µl acetaldehyde] was added and incubated at room temperature for 24 hr [30]. The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$DNA\ \ fragmentation = \frac{OD\ of\ fragmented\ DNA(S)}{OD\ of\ fragemented\ DNA(S) + OD\ of\ intact\ DNA(P)}$$

Biochemical analysis

Analysis of oxidative stress:

Recent evidence has shown that oxidative stress may play a role in the pathogenesis of autoimmune diseases, and this is an issue of considerable research interest in the field of genotoxicity. Therefore, we evaluated both the relationship between Glyphosate and serum levels of certain indicators of oxidative stress such as Malondialdehyde (MDA). Liver tissues were homogenized in 20mm Tris-HCI (pH 7.4). Homogenates were centrifuged at 6000 *g* for 30 min. Malondialdehyde (MDA) levels in the supernatants were determined using a spectrophotometric assay kit according to the manufacturer's instructions. The absorbance of the resultant pink product was measured at 534 nm [31]. The lipid peroxidation values were expressed as nm MDA/mg tissue.

Statistical analysis:

Data obtained were subjected to one way analysis of variance (ANOVA) using SAS, Version 8.2 [32], followed by Duncan Multiple range test for comparison between groups.

Results

Detection of DNA damage using comet assay:

The present results using comet assay showing the values of tail length and moment obtained from comet assay in mice treated with Glyphosate pesticide and / or orange juice (Table 1). It is clear that there was a highly significant increase in the tail length in blood cells of mice treated with Glyphosate compared to the control ones especially for the long period treatment (4 weeks) that clearly indicates a genotoxic effect of pesticide. Data in Table (1) revealed that orange juice treatment in combination with pesticide decreased the values of DNA damage compared to treated mice with pesticide alone. The protective effect of orange juice was observed either for the short or long period treatment, indicating a protective effect of orange juice against DNA damage induced by Glyphosate pesticide.

Treatment	No. of animals	2 weeks	4 weeks
Control	10	1.0 ± 1.00^{d}	1.00 ± 1.000^{d}
Orange Juice	10	7.80 ± 0.437^{c}	12.8 ± 0.441^{c}
Glyphosate	10	27.8 ± 0.438^{a}	39.8 ± 0.435^{a}
Glyphosate + Orange juice	10	24.8 ± 0.436^{b}	22.88 ± 0.437^{b}

Table 1 **DNA damage frequency in mice leucocytes exposed to Glyphosate and/or orange juice for 2 and 4 weeks.** Values are expressed as means S.E.; Different small superscript letters are differing significantly.

Micronucleus formation

The effect of Glyphosate pesticide and orange juice for 2 and 4 weeks treatment on MnPCEs formation in the bone marrow cells of male mice is summarized in Tables 2 and 3. The results revealed that MnPCEs formation in control mice was lower than those in all treated groups. Treatment of male mice with Glyphosate increased significantly the formation of MnPCEs compared to the control group either after 2 or 4 weeks treatment (Tables 2 and 3).

On the other hand, treatment of male mice with orange juice alone revealed similar rate of MnPCEs formation compared to that in control group (Tables 2 and 3). Moreover, treatment of male mice with orange juice combined with Glyphosate decreased significantly the incidence of MnPCEs compared to Glyphosate alone either after 2 or 4 weeks treatment.

Biohelikon

Treatment		Mn PCEs/2000 PCE		NCE screened	Ratio	PCE/NCE
	PCE screened	Number	Mean±SD			Mean±SD
Control	2000	3	5.40±2.302 ^{c}	672	2.97	3.29±0.477
	2000	3		681	2.93	
	2000	8		506	3.95	
	2000	7		547	3.65	
	2000	6		676	2.95	
Orange juice	2000	3	5.60±2.607^{c}	673	2.97	2.99±0.223
	2000	9		745	2.68	
	2000	7		605	2.30	
	2000	6		650	3.07	
	2000	3		675	2.96	
Glyphosate	2000	19	17.40 ±1.140^{a}	748	2.67	3.63±1.258
	2000	16		387	5.16	
	2000	17		412	4.85	
	2000	17		722	2.77	
	2000	18		737	2.71	
	2000	19		748	2.67	
Orange juice + Glyphosate	2000	14	12.80±1.303 ^{b}	474	4.21	3.77±1.003
	2000	11		401	4.98	
	2000	13		766	2.61	
	2000	12		699	2.86	
	2000	14		475	4.21	

Table 2 **Effect of 2 weeks treatment of Glyphosate and/or orange juice on Micronuclei formation in bone marrow cells of mice** PCE: Polychromatic erythrocytes; NCE: Normochromatic erythrocytes.; Values are expressed as means S.E.; Different small superscript letters are differing significantly.

Treatment		Mn PCEs/2000 PCE		NCE screened	Ratio	PCE/NCE
	PCE screened	Number	Mean±SD			Mean±SD
Control	2000	3	5.40±2.302 ^{c}	672	2.97	3.29±0.477
	2000	3		681	2.93	
	2000	8		506	3.95	
	2000	7		547	3.65	
	2000	6		676	2.95	
Orange juice	2000	3	5.80 ±2.387 ^{c}	674	2.96	2.99±0.223
	2000	9		745	2.68	
	2000	7		605	2.30	
	2000	6		650	3.07	
	2000	3		675	2.96	
Glyphosate	2000	28	28.40±1.140 ^{a}	523	3.82	3.33±0.451
	2000	27		529	3.78	
	2000	30		656	3.04	
	2000	28		712	2.80	
	2000	29		614	3.25	
	2000	28		523	3.82	
Orange juice + Glyphosate	2000	24	23.60±0.89 ^{b}	556	3.59	3.50±0.699
	2000	25		687	2.91	
	2000	23		432	4.62	
	2000	23		687	2.91	
	2000	23		569	3.51	

Table 3 **Effect of 4 weeks treatment of Glyphosate and/or orange juice on Micronuclei formation in bone marrow cells of mice** PCE: Polychromatic erythrocytes; NCE: Normochromatic erythrocytes.; Values are expressed as means S.E; Different small superscript letters are differing significantly.

Chromosome aberrations in somatic cells:

Cytogenetic analysis in the present study showed that there were structural and numerical chromosomal aberrations (Tables 4 and 6). Structural aberrations consisted of gaps, breaks, fragments, centromeric attenuations and centric fusion. Numerical aberrations were hypoploidy (2n-1) and hyperploidy (2n+1). The results shown in Tables 4 and 6 revealed that there was a significant difference between pesticide treated mice and the control group in all types of structural and numerical aberrations either upon pesticide treatment for 2 or 4 weeks.Addition of orange juice to pesticide resulted in a significant decrease in all types of chromosomal aberrations (except for break in the 2 weeks treated group) compared to pesticide treated group. There was a slight increase in all types of chromosomal aberrations in the pesticide treated group for 4 weeks than the 2 weeks treated counterpart but the treatment with orange juice significantly decreased all types of aberrations as well.

Treatment	No. of animals	No. of examined cells	Structural aberrations						Numerical aberrations	
			Gap	Break	Fragment	Centromeric attenuation	Centric fusion	Total abenration	Hypoploidy	Hyperploidy
Control	10	500	0.20±0.44^{a}	0.20±0.44^{a}	0.40±0.54^{a}	0.80±0.44^{a}	0.20±0.44^{a}	1.80±1.48^{a}	0.20±0.44^{a}	0.00±0.00^{a}
Orange juice	10	500	0.60±0.54^{a}	0.40±0.54^{a}	0.40±0.54^{a}	0.20±0.44^{a}	0.80±0.83^{a}	2.40±1.94^{b}	0.00±0.00^{a}	0.00±0.00^{a}
Glyphosate	10	500	5.80±0.83^{c}	7.20±0.44^{b}	5.00±.70^{c}	10.40±0.89^{c}	7.40±0.89^{c}	30.80±0.83^{d}	1.60±0.54^{b}	2.20±0.44^{c}
Glyphosate + Orange juice	10	500	3.00±0.70^{b}	2.40±0.54^{b}	2.20±0.44^{b}	4.40±0.54^{b}	4.20±0.44^{b}	16.20±1.09^{c}	0.40±0.54^{a}	1.60±1.14^{b}

Table 4 **Mean values of different chromosomal aberrations in bone marrow of mice treated with Glyphosate and/or orange juice for 2 weeks.** Values are expressed as means S.E.; Different small superscript letters are differing significantly.

Chromosome aberrations in germ cells:

Chromosome aberration in seprmatocytes treated for 2 and 4 weeks (Tables 5 and 7, respectively), consisted of chain, autosomal and X-y univalent, beside the numerical aberration (n±1). The present results showed that pesticide treated group had more frequencies of chromosome aberrations than the control group and that is true for both 2 and 4 weeks of treatment. Addition of orange juice to pesticide treated group decreased the frequency of such aberration in the two periods of treatment.

The frequency of aberrations in the 4 weeks pesticide treated group was high compared to the 2 weeks of treatment (except for x-y univalent) but the treatment with orange juice significantly decreased such types of aberrations.

Treatment	No.of animals	No. of examined cells	Structural aberrations			Total aberrations	Numerical aberrations	
			Chain	Univalent			N+1	N-1
				Autosomal	X-y			
Control	10	500	0.20±0.44^{a}	0.40±0.54^{a}	0.40±0.54^{a}	1.00±1.00^{a}	0.20±0.44^{a}	0.20±0.44^{a}
Orange juice	10	500	0.20±0.44^{a}	0.40±0.54^{a}	0.20±0.44^{a}	0.80±0.83^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Glyphosate	10	500	2.20±0.44^{c}	4.80±1.48^{c}	7.80±1.30^{c}	14.80±1.78^{c}	1.40±1.14^{b}	2.20±0.44^{b}
Glyphosate + Orange juice	10	500	1.60±0.54^{b}	3.60±0.54^{b}	2.20±0.44^{b}	7.40±0.54^{b}	0.40±0.54^{a}	0.40±0.54^{a}

Table 5 **Mean values of different chromosomal aberrations in spermatocytes of mice treated with Glyphosate and/or orange juice for 2 weeks.** Values are expressed as means S.E.; Different small superscript letters are differing significantly.

Treatment	No.of animals	No. of examined	Structural aberrations						Numerical aberrations	
			Gap	Break	Fragment	Centromeric attenuations	Centric fusion	Total aberrations	N+1	N-1
Control	10	500	0.20±0.44^{a}	0.20±0.44^{a}	0.40±0.54^{a}	0.20±0.44^{a}	0.60±0.54^{a}	1.60±1.14^{a}	0.20±0.44^{a}	0.20±0.44^{a}
Orange juice	10	500	0.40±0.54^{a}	0.60±0.54^{a}	1.00±0.00^{b}	2.20±0.44^{b}	1.00±0.00^{b}	5.40±0.89^{b}	0.00±0.00^{a}	0.20±0.44^{a}
Glyphosate	10	500	6.60±1.34^{c}	5.60±0.54^{c}	7.80±0.83^{d}	13.80±0.83^{d}	15.00±0.70^{d}	48.80±2.38^{d}	1.60±1.14^{b}	3.20±0.70^{b}
Glyphosate + Orange juice	10	500	2.40± 0.89 ^{b}	1.60±0.54^{b}	2.00±0.00^{c}	4.20±0.44^{c}	3.0±0.70^{c}	13.20±1.0^{c}	0.60±0.54^{a}	0.40±0.54^{a}

Table 6 **Mean values of different chromosomal aberrations in bone marrow of mice treated with Glyphosate and/or orange juice for 4 weeks.** Values are expressed as means S.E.; Different small superscript letters are differing significantly.

Treatment	No. of animals	No. of examined cells	Structural aberrations			Total aberrations	Numerical aberrations	
			Chain	Univalent			N+1	
				Autosomal	X-y			N-1
Control	10	500	0.20±0.44^{a}	0.40±0.54^{a}	0.20±0.44^{a}	0.80±1./30^{a}	0.20±0.44^{a}	0.20±0.44^{a}
Orange juice	10	500	0.40±0.54^{a}	0.20±0.44^{a}	0.40±0.54^{a}	1.00±0.70^{b}	0.00±0.00^{a}	0.20±0.44^{a}
Glyphosate	10	500	5.0±0.70^{c}	11.60±1.140^{c}	6.60±0.54^{c}	23.20±1.09^{d}	2.40±0.54^{b}	1.40±0.54^{b}
(Glyphosate + Orange juice	10	500	1.00±0.00^{b}	1.40±0.54^{b}	2.40±0.54^{b}	4.80±0.44^{c}	0.40±0.54^{a}	0.60±0.54^{a}

Table 7 **Mean values of different chromosomal aberrations in spermatocytes of mice treated with Glyphosate and/or orange juice for 4 weeks.** Values are expressed as means S.E.; Different small superscript letters are differing significantly.

DNA fragmentation

The present study was performed to evaluate the effect of Glyphosate and/or orange juice by determination of the DNA fragmentation in male mice. The fragmented DNA was examined in liver tissues collected from treated animals using diphenylamine reaction procedure described in the Methods section. The results (Table 8) revealed that fragmented DNA in untreated control animals was lower than all other treated groups. However, rate of DNA fragmentation in animals treated with Glyphosate was markedly higher than control mice either for 2 or 4 weeks treatment.

Treatment	**DNA Fragmentation** (M±S.E.) **2 week**	**DNA Fragmentation** (M ±S.E.) **4 week**
Control	7.24±0.246 ^{c}	7.24±0.246 ^{c}
Orange juice	7.84±0.408 ^{c}	8.80±0.374 ^{c}
Glyphosate	28.80±0.374^{a}	41.86±0.428 ^{a}
Orange juice + Glyphosate	22.33±0.276 ^{b}	34.88±0.446 ^{b}

Table 8 **DNA Fragmentation in mice treated with Glyphosate and/ or orange juice** Values are expressed as means S.E.; Different small superscript letters are differing significantly.

Treatment	**Malondialdehyde (MDA) (nmol/g tissue)** (M±S.E) 2week	**Malondialdehyde (MDA) (nmol/g tissue)** (M±S.E.) 4 week
Control	0.60±0.0244 ^{c}	0.60±0.244 ^{c}
Orange juice	0.70±0.200 ^{c}	0.80±0.200 ^{c}
Glyphosate	19.90±0.458 ^{a}	26.86±0.423 ^{a}
Orange juice + Glyphosate	11.84±0.408 ^{b}	17.80±0.374 ^{b}

Table 9 **Effect Glyphosate and/or orange juice on Malondialdehyde (MDA) in mice.** Values are expressed as means S.E.; Different small superscript letters are differing significantly.

On the other hand, treatment of male mice with orange juice alone revealed low DNA fragmentation which was relatively similar to control rats. In addition, treatment of male mice with Glyphosate combined with orange juice decreased DNA fragmentation occurred due to Glyphosate treatment either for 2 or 4 weeks treatment (Table 8).

Analysis of oxidative enzyme malondialdehyde:

The results of biochemical analysis revealed a significant increase in the mean values of Malondialdehyde (MDA) in Glyphosate treated mice either for 2 or 4 weeks compared to control. However, the combination of Glyphosate pesticide with orange juice significantly decreased the mean values of these parameters either for 2 or 4 weeks of treatment indicating the protective effects of orange juice against the toxicity of Glyphosate.

Discussion

The results of the present study reveals that Glyphosate intake caused significant incidence of DNA damage, chromosomal aberrations and induction of micronuclei as well as increased the levels of MDA in a dose- and time-dependent manner. Various cytogenetic results on commercial Glyphosate are problematic. They may depend on purity of the active agent and on the nature of inert components. Surfactants and other inert compounds were previously suggested to increase the toxicity of the herbicide [33]. In a recent study, *Caiman latirostris* embryos were exposed at early embryonic stage to different sub lethal concentrations of Roundup (range from 50–1750 g/egg), and the results from both the comet assay and the MN test revealed a concentration dependent effect [3].

Glyphosate reported for positive clastogenic and genotoxic effects *in vitro* [34] which are consistent with our genotoxic results. DNA damage and chromosomal aberrations (CAs) are considered to detect early effects of xenobiotic insult and evaluation of the frequency of CAs is a sensitive cytogenetic assay for detecting exposure to mutagens and carcinogens [35]. In the present study, Glyphosate induced CAs could be attributed to early changes either an increase in induced DNA lesions or interference with their repair. Glyphosate has been reported to cause DNA damage in erythrocytes of bullfrog tadpoles (*R. catesbeiana*) [36]. However, few studies reported that Glyphosate is weak or non-clastogenic *in vivo* [37].

The MN induction assay was used as an additional sensitive biological indicator of the damage to somatic cell genome of subjects exposed to pesticide mixtures occupationally. It is known that the appearance of MN is related to the loss of chromosome fragments due to chromosome breaks [38]. Our results revealed that there was elevation in the number of micronuclei in the Glyphosate exposed animals. Because MN could be the consequence of the mitotic spindle malfunction, it is possible that the Glyphosate could also express an aneugenic mode of action as inhibiting cell division and mitotic spindle apparatus.

The molecular mechanisms responsible for the genotoxicity of Glyphosate are not clarified yet. However, the CAs and the micronucleus formation observed in animals clearly indicate that these compounds interact with chromatin DNA and induce damage there. Such interactions/DNA damage may be caused by an increased incidence of alkali labile

Biohelikon

sites in DNA as observed in kidney and liver with Glyphosate treatment in CD-1 mice [39]. Alkali labile sites are generally produced at basic sites in DNA and may be revealed under conditions that denature DNA secondary structure.

On the other hand, the combination of orange juice with Glyphosate reduced that elevation in these parameters and that coincide with the findings of Cavuşoğlu et al [16]. The protective effect of orange juice could be attributed to its antioxidant properties.

There are many studies evaluating the antioxidant potential oforange juice, mostly *in vitro*. All *in vitro* studies have shown thatorange juice has considerable antioxidant potential. The high content of flavonones is linked to orange juice's antioxidant potential. Juices rich in flavonones are the second-best antioxidant, after fruits rich in anthocyanins (red, purple, or blue fruits) [40,41]. Certain factors affect in vitro antioxidant potential. High temperatures during thermal treatment or during storage (> 20 °C) and long-term storage (> 4 mo) decrease antioxidant activity due to phytochemical composition changes [42,43]. Sweetening seems to lead to the extinction of most antioxidant activity [19]. To date, it is not clear how much each orange juice constituent con-tributes to the overall antioxidant activity. It is possible that some compound act at short-term losing their antioxidant capacity (e.g., vitamin C) and other such as phenolics might retain their antioxidant potential for longer periods (e.g., phenols are stable for long periods but change in term of their relative components) [44,45].

Orange juice was more effective as co-treatment and was most potent inhibitor in relation to other substances tested [46]. Riso et al. [47] showed that lymphocytes of individuals supplemented with blood OJ (600 mL× 21 days) had increased resistance to H2O2 -mediated DNA damage, as evaluated by the comet assay. In mice, our data indicate that orange juice reduced the genotoxicity induced by Glyphosate, also using the comet assay. Differences were observed depending on the treatment schedule (orange juiceas pre- or post-treatment). Orange juice, reduced the DNA damage. Guarnieri et al. [48] evaluated the effect of the intake of a single portion of blood orange juice(300 mL) on mono nuclear blood cell resistance to H2O2 -induced DNA damage [48] by the comet assay.

Conclusion

Glyphosate appears to have the ability to cause genetic damage for both somatic (bone marrow) and germ (spermatocyte) cells, that may lead to cancer. This conclusion is supported by the epidemiological reports of DNA damage in people exposed to Glyphosate, and cases of lymphocytic cancer. Furthermore, the possibility of the link between Glyphosate and the biochemical alterations [16] support these findings. As well, Glyphosate was found to decreased cell viability in a dose dependent manner [49,50], and altered gene expression in the cells [51]. Nevertheless, glyphosate's harmful effects were counterbalanced in a time-dependent manner by the protective capacity of orange juice, probably through its antioxidant properties.

Data showed some differences in the protective capacity of the orange juice. Orange juice exhibited a time-dependent protective effect, with higher effects in cells chromosomal aberrations as soon as 2 weeks, potentially via its antioxidant properties (evaluated by the MDA oxidative stress assay).

References

1. van der Werf H M G (1996). Assessing the impact of pesticides on the environment. *Agriculture, Ecosystems and Environment*, vol 60, 81-96.
2. Simoniello MF, Kleinsorge EC, Scagnetti JA, Grigolato RA, Poletta GL, *et al.* (2008). DNA damage in workers occupationally exposed to pesticide mixtures. *J Appl Toxicol*, 28(8): 957-965.
3. Poletta G L, Larriera A, Kleinsorge E, Mudry M D (2009). Genotoxicity of the herbicide formulation Roundup® (Glyphosate) in broad-snouted caiman (Caiman latirostris) evidenced by the Comet assay and the Micronucleus test *Mutation Research*, vol 672, no 2, pp 95-102.
4. Lander BF, Knudsen LE, Gamborg MO, Järventaus H, Norppa H (2000). Chromosome aberrations in pesticide-exposed greenhouse workers. *Scand J Work Environ Health*, 26(5): 436-442.
5. Meinert R, Schüz J, Kaletsch U, Kaatsch P, Michaelis J (2000). Leukemia and non-Hodgkin's lymphoma in childhood and exposure to pesticides: results of a register-based case-control study in Germany. *Am J Epidemiol*, 151(7): 639-646.
6. Ji BT, Silverman DT, Stewart PA, Blair A, Swanson GM, *et al.* (2001). Occupational exposure to pesticides and pancreatic cancer. *Am J Ind Med*, 39(1): 92-99.
7. Zhao S, Narang A, Gierthy J, Eadon G (2002). Detection and characterization of DNA adducts formed from metabolites of the fungicide ortho-phenylphenol. *J Agric Food Chem*, 50(11): 3351-3358.
8. Blair A, Zahm SH (1995). Agricultural exposures and cancer. *Environ Health Perspect*, 103 Suppl 8: 205-208.
9. Webster LR, McKenzie GH, Moriarty HT (2002). Organophosphate-based pesticides and genetic damage implicated in bladder cancer. *Cancer Genet Cytogenet*, 133(2): 112-117.
10. Clary T, Ritz B (2003). Pancreatic cancer mortality and organochlorine pesticide exposure in California, 1989-1996. *Am J Ind Med*, 43(3): 306-313.
11. PANAP (2009). Glyphosate: Pesticide Action Network Asia and the Pacific. *Empowering People for Change*,
12. Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, *et al.* (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology*, 262(3): 184-191.
13. Manas F, Peralta L, Raviolo J, Garcia Ovando H, Weyers A (2009). Genotoxicity of Glyphosate assessed by the comet assay and cytogenetic tests. *Environ Toxicol Pharmacol*, 28:37-41.
14. Lioi MB, Scarfì MR, Santoro A, Barbieri R, Zeni O, *et al.* (1998). Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro. *Mutat Res*, 403(1-2): 13-20.
15. SivikovÃ¡ K, Dianovská»³ J (2006). Cytogenetic effect of technical glyphosate on cultivated bovine peripheral lymphocytes. *Int J Hyg Environ Health*, 209(1): 15-20.
16. Cavusoglu, K, Yapar, K, Oruc, E, Yalsin, E (2011). Protective effect of Ginkgo biloba L. leaf extract against Glyphosate toxicity in Swiss albino mice. *J Med Food*, 14(10): 1263-1272.
17. Morton J (1987). Orange . In:Fruits of Warm Climates, J.F. Morton (ed.). *Miami,FL: Florida Flair Books*, 134-142.2.
18. Moufida S, Marzouk B (2003). Biochemical characterization of blood orange, sweet orange, lemon, bergamot and bitter orange. *Phytochemistry*, 62(8): 1283-1289.
19. Fiore A, La Fauci L, Cervellati R, Guerra MC, Speroni E, *et al.* (2005). Antioxidant activity of pasteurized and sterilized commercial red orange juices. *Mol Nutr Food Res*, 49(12): 1129-1135.
20. Rampersaud GC (2007). A comparison of nutrient density scores for 100% fruit juices. *J Food Sci*, 72(4): S261-266.
21. Franke SIR, Ckless K, Silveira JD , Rubensam G, Brendel M, et al (2004). Studyof antioxidant and mutagenic activity of different orange juices. *Food Chem*, 88, 45-55.
22. Franke AA, Cooney RV, Henning SM, Custer LJ (2005). Bioavailability and antioxidant effects of orange juice components in humans. *J Agric Food Chem*, 53(13): 5170-5178.

23. Vieira SM, Theodoro KH, and Gloria MBA (2007). Profile and levels of bioac-tive amines in orange juice and orange soft drink. *Food Chemistry*, 100, 895-903.
24. O'Neil CE, Nicklas TA, Rampersaud GC, Fulgoni VL 3rd (2012). 100% orange juice consumption is associated with better diet quality, improved nutrient adequacy, decreased risk for obesity, and improved biomarkers of health in adults: National Health and Nutrition Examination Survey, 2003-2006. *Nutr J*, 11: 107.
25. Fairbairn DW, Olive PL, O'Neill KL (1995). The comet assay: a comprehensive review. *Mutat Res*, 339(1): 37-59.
26. Collins A, Dusinská M, Franklin M, Somorovská M, Petrovská H, *et al.* (1997). Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ Mol Mutagen*, 30(2): 139-146.
27. Adler ID (1984). Cytogenetic tests in mammals. *Venitt S, Parry JM (eds) Mutagenicity testing (a practical approach). IRI Press, Oxford*, 275-306.
28. Preston RJ, Dean BJ, Galloway S, Holden H, McFee AF, *et al.* (1987). Mammalian in vivo cytogenetic assays. Analysis of chromosome aberrations in bone marrow cells. *Mutat Res*, 189(2): 157-165.
29. Brewen, J G, Preston, R J (1978). Analysis of chromosome aberrations in mammalian germ cells. *A. Holloendr and F J de. Serres (Eds.) chemical Mutagens, Vol. 5, plenum, New York*, 127-150.
30. Lu T, Xu Y, Mericle MT, Mellgren RL (2002). Participation of the conventional calpains in apoptosis. *Biochim Biophys Acta*, 1590(1-3): 16-26.
31. Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Annals of Biochemistry*, 95:351-358.
32. SAS (2001). SAS/STAT User's Guide, Version 8.2 *SAS Institute Inc, Cary, NC, USA*,
33. Cox C (1999). Glyphosate (Roundup), in Global Pesticide Campaigner *Pesticide Action Network (PAN): North America, San Francisco, Calif, USA*, 12-19.
34. Lioi M B, Scarfi M R, Santoro A, *et al.* (1998). Cytogenetic damage and induction of pro-oxidant state in human lymphocytes exposed in vitro to gliphosate, vinclozolin, atrazine, and DPX-E9636. *Environmental and Molecular Mutagenesis*, 32:(1) 139-146.
35. Bonassi S, Abbondandolo A, Camurri L, *et al.* (1995). Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study. *Cancer Genetics and Cytogenetics*, 79:(2) 133-135.
36. Clements C, Ralph S, Petras M (1997). Genotoxicity of select herbicides in Rana catesbeiana tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. *Environ Mol Mutagen*, 29(3): 277-288.
37. Dimitrov BD, Gadeva PG, Benova DK, Bineva MV (2006). Comparative genotoxicity of the herbicides Roundup, Stomp and Reglone in plant and mammalian test systems. *Mutagenesis*, 21(6): 375-382.
38. Heddle JA, Cimino MC, Hayashi M, Romagna F, Shelby MD, *et al.* (1991). Micronuclei as an index of cytogenetic damage: past, present, and future. *Environ Mol Mutagen*, 18(4): 277-291.
39. Peluso M, Munnia A, Bolognesi C, Parodi S (1998). 32P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. *Environ Mol Mutagen*, 31(1): 55-59.
40. Proteggente AR, Pannala AS, Paganga G, Van Buren L, Wagner E, *et al.* (2002). The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radic Res*, 36(2): 217-233.
41. Del Caro A, Piga A, Vacca V, and Agabbio M (2004). Changes of flavonoids, vitaminC and antioxidant capacity in minimal lyprocessed citrus segments and juices during storage. *Food Chemistry*, 84, 99-105.
42. LoScalzo R, Iannoccari T, Summa C, Morelli R, Rapisarda P (2004). Effect of thermal treatments on antioxidant and antiradical activity of blood orange juice. *Food Chemistry*, 85, 41-47.
43. Klimcza k I, Malcka M, Szlachta M, Gliszczynska-Swiglo A (2007). Effectof storage on the content of polyphenols, vitamin C and the antioxidantactivity of orange juices. *J Food Compost Anal*, 20, 313-322.
44. Sentandreu E, Navarro JL, and Sendra JM (2007). Effect of technological processes and storage on flavonoids content and total cumulative fast-kinetics and cumulative slow-kinetics antiradical activities of citrus juices. *Eur Food Res Technol*, 225, 905-912.
45. Tabart J, Kevers C, Pincemail J, Defraigne JO, and Dommes J (2009). Compar-ative antioxidant capacities of phenolic compounds measured by varioustests. *Food Chemistry*, 113, 1226-1233.
46. He Y, Campbell TC (1990). Effects of carotenoids on aflatoxin B1-induced mutagenesis in S. typhimurium TA 100 and TA 98. *Nutr Cancer*, 13(4): 243-253.
47. Riso P, Visioli F, Gardana C, Grande S, Brusamolino A, *et al.* (2005). Effects of blood orange juice intake on antioxidant bioavailability and on different markers related to oxidative stress. *J Agric Food Chem*, 53(4): 941-947.
48. Guarnieri S, Riso P, Porrini M (2007). Orange juice vs vitamin C: effect on hydrogen peroxide-induced DNA damage in mononuclear blood cells. *Br J Nutr*, 97(4): 639-643.
49. Reyes N, Martinez A, Reyes I, Geliebter J (2006). Molecular and cellular effects of Glyphosate on human lymphocytes: implications for non-Hodgkin's lymphoma. *Proc 97th Ann Meet Amer Assoc Canc Res Abs*,
50. Martínez A, Reyes I, Reyes N (2007). [Cytotoxicity of the herbicide glyphosate in human peripheral blood mononuclear cells]. *Biomedica*, 27(4): 594-604.
51. Reyes N, Martinez A, Reye I, Geliebter J (2007). Differential effects of Glyphosate and Roundup in gene expression of human peripheral blood mononuclear cells: Implications for hematological carcinogenesis *Proc 98th Ann Meet Amer Assoc Canc Res, Abs*,