Research Article

MONONUCLEAR LEUKOCYTE DNA DAMAGE ON HIGHER CELLS CAUSED BY ECO-FRIENDLY PESTICIDES AND THEIR ANALYSIS USING CASP® PROGRAMME

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ABSTRACT

The common use of pesticides for domestic and agricultural purpose is a major concern for public health. Since many *in vitro* studies revealed that the use of pesticides had genotoxic effects on human health and targeted organisms, the use of environmentally friendly pesticides aimed to prevent or reduce the negative effects of those chemicals on non-targeted organisms. In the present study, the genotoxicity of so called eco-friendly pesticides such as dimethoate (400-, 200 µg ml^{-1}), methyl parathion (360-, 180 µg ml^{-1}) and alphacypermethrin (100-, 50 µg ml^{-1}) was evaluated with their corresponding advised and low doses, respectively, on the induction of DNA damage in human peripheral blood lymphocytes (PBL) using the single cell gel electrophoresis assay (SCGE assay or comet assay) immediately after 30 min of treatment for the mononuclear leukocyte DNA damage. The chemicals tested on whole blood samples showed that the advised or even lower doses of each pesticide caused extensive DNA damage. The expression of DNA damage was also compared with that of CASP programme. **Key Words:** Alkaline single cell gel elecrophoresis, Comet analysis, DNA damage, Pesticides, CASP programme

ÇEVRE DOSTU PESTİSİTLERİN YÜKSEK YAPILI HÜCRELERDE NEDEN OLDUĞU TEK HÜCRE LÖKOSİT DNA HASARI VE CASP PROGRAMI İLE ANALİZİ

ÖZET

Tarımsal alanlarda ve evlerimizde yaygın olarak kullanılan pestisitler halk sağlığı için büyük önem arz etmektedir. Pestisit kullanımının hedef organizmalar ve insan sağlığı üzerine genotoksik etkilerinin olduğu birçok *in vitro* çalışma ile ortaya konulması sebebiyle, bu kimyasalların hedef olmayan organizmalar üzerinde meydana gelen olumsuz etkilerini önlemek ya da azaltmak için çevre dostu pestisitlerin kullanılması hedeflenmiştir. Bu çalışmada, çevre dostu olarak bilinen pestisitlerden dimethoate (400-, 200 µg ml^{-1}), methyl parathion (360-, 180 µg ml^{-1}) and alphacypermethrin (100-, 50 µg ml^{-1})'in tavsiye edilen ve düşük dozlarının insan periferal kan hücrelerindeki DNA hasar oluşumu üzerine genotoksik etkileri araştırılmıştır. Bu amaçla, kan örneklerine 30 dk süresince farklı dozlarda çevre dostu pestisitler uygulandıktan hemen sonra tek hücre jel elektroforezi (SCGE ya da comet analizi) kullanılarak, tek çekirdekli lökosit DNA hasarı incelenmiştir. Sonuçlar bu kimyasalların tavsiye edilen ya da tavsiye edilenden daha düşük dozlarının bile büyük oranda DNA hasarına neden olduğunu göstermiştir. Elde edilen DNA hasarının durumu CASP programı kullanılarak kıyaslama yoluna gidilmiştir.

Anahtar Kelimeler: Alkali tek hücre jel elektroforez, Comet analizi, DNA hasarı, Pestisitler, CASP pogramı

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® The base model for CASP (Comet Assay Software Project) programme was developed by K. Konca et al., 2003.

INTRODUCTION

Changes in agricultural practices have resulted in a dramatic increase in the use of pesticides over the past 40 years. These agrochemicals have been intensively used in agriculture to improve crop production and protect stored crops. However, health risks have been suggested in human populations that are occupationally exposed to these agrochemicals (Lebailby et al., 1998). Pesticides, in general, are very toxic chemicals intended to control pest populations although the benefits associated with their use in agriculture are unquestionable, many of their active substances have potentially negative effects on human health. The use of pesticides especially overdose applications has been known as carcinogenic substances (Garret et al., 1986; Sinha, 1989). Many of them carry risk factors such as lung cancer (Barthel, 1981), bladder cancer (Viel and Chalier, 1995), and leukemia (Blair and Zahm, 1995) in workers exposed to pesticides. If pesticides are intend to heavily control the targeted organisms their common mode of action is to generate toxicity in their metabolism, however, this also affects the beneficial organisms as well as human beings. The widespread use of pesticides for preventing, destroying or repelling pests has resulted in anxiety about the possible hazards to public health (WHO, 1988; Ecobichon, 2001). The most commonly used pesticides in our country for agricultural purposes are dimethoate and methyl parathion, the carbamates pirimicarb, and the pyrethroids cypermethrin. Although understanding of the biological effects of currently used pesticides has increased in recent years, there are often incomplete, and sometimes contradictory data on their genotoxicity (Dimitrov et al., 2006; Cavas and Konen, 2007). Yet, the results are sometime inconsistent, for example, a variety of *in vitro* and *in vivo* assays have been used to evaluate the induction of DNA damage and mutation by pesticides. The *Saccharomyces cerevisiae* D7 and *Salmonella typhimurium* (TA98) mutation assays, as well as the micronucleus (MN) assay in lymphocytes, were the most commonly used tests (Garaj-Vrhovac and Zeljezic, 2000; Lucero et al., 2000) to examine the mutagenicity and genotoxicity of various pesticides. However, many genotoxicity tests are not satisfactory and time consuming to find out the genotoxic effects of those substances. For example, dimethoate was found non-mutagenic in *Salmonella typhimurium* TA 100, but not found mutagenic to *S. typhimurium* TA 1535. Again, methyl parathion resulted in an increase the number of sister chromatid exchange (SCE) and choromosomal aberration (CA) in fish and rats, induced micronuclei in mice (Das and John 1999; Undeger et al. 2000), but it was unable to chromosome mutations in *Drosophila melanogaster* (Velazquez et al. 1990). Likewise, many other chemicals such as carbendazim and benomyl caused toxicity in higher doses.

The increased use of pesticides worldwide have led to a general agreement on the need of further biological and genotoxical studies on the adverse effect of these chemicals on human health and non-targeted organisms (Weisenburger, 1993). In recent years, environmentally friendly pesticides have also been on the market, however, considering the the long-term use of pesticides, the risk for cancer would become the most common disease factor. Because these stress agents could have deleterious effects on metabolism of living organisms, in addition, the harmful effects could also be observed on their DNA. If the damage of DNA cannot be repaired, then the cell would not function properly and eventually die. In this case, the organism would either kill its own cell, named as apoptosis, or try to repair it. However, if this could not be achieved, cancer would be inevitable (Kocyigit et al., 2005; Dikilitas et al., 2009).

Exposure to pesticides may occur during mixing, spraying or application or during cleaning and repair of equipment or during early re-entry into fields (WHO, 1993). Since these chemicals contain toxic substances, it is harmul to the users and non-target organisms as well. For example, dimethoate is moderately toxic by ingestion, inhalation and dermal absorption. As with all organophosphates, dimethoate is readily absorbed through the skin. Persons with respiratory ailments, recent exposure to cholinesterase inhibitors, impaired cholinesterase production, or with liver malfunction may be at increased risk from exposure to dimethoate. High environmental temperatures or exposure of dimethoate to visible or UV light may enhance its toxicity (Meister, 1992). Methyl parathion is also highly toxic by inhalation and moderately toxic by dermal adsorption. Although methyl parathion has a short half-life (1 hour on cotton) when applied to crops, the risk of

exposure to agricultural workers is low. However, pesticide users who handle large quantities of concentrated methyl parathion are at a higher risk (Hayes and Laws, 1990). It is rapidly absorbed into the bloodstream through all normal routes of exposure. The symptoms of pesticide may appear within 1 to 2 hours. Alphacypermethrin, on the other hand, is a synthetic pyrethroid insecticide used to control many pests, including moth pests of cotton, fruit and vegetable crops (Hayes and Laws, 1990).

Repeated or prolonged exposure to these chemicals may result in the same effects as acute exposure. However, only the symptoms might delay. Although these chemicals have been reported that they are not carcinogenic or mutagenic but tests with very high doses on mice caused a temporary increase in the number of bone marrow cells with micronuclei (Howard, 1989).

However, the genetical aspects of these chemicals and many other chemicals have not been easily measured due to the technical problems caused by either characteristics of test materials or lack of technical facilities. Sometimes, nature friendly pesticides are advised to be used in large quantities, however, their advised doses or even lower doses could be harmful to the non-targeted organisms. Assays to quick determination and measurement of the genotoxicity of these chemicals are quite important. *In vitro* toxicology studies have forced us to use a reliable *in vitro* assay to detect the cytotoxicity of pesticides and other toxic chemicals. The alkaline single cell gel electrophoresis technique or comet assay is a relatively new and sensitive assay that measures single-strand breaks, double strand breaks, alkali labile sites (primarily apurinic and apyrimidinic sites), incomplete excision repair sites, and DNA cross-links in individual cells (Collins, 2002; Gichner et al., 2009; Dikilitas et al., 2009). The use of comet assay, a highly regarded toxicology assay, has been extensively used as a research tool in genotoxicity. The assay method is a sensitive and straightforward for measuring DNA damage in individual cells (Ostling and Johanson, 1984; Singh et al., 1988).

In this study, the genotoxic effects of commonly used nature friendly pesticides, dimethoate and methyl parathion and alphacypermethrin were evaluated at advised doses by the prescription of the company. The lower doses than those of advised doses were also tested on blood cells to find out the existence of genotoxic effects on human lymphocytes. The results of DNA fragments (damage) obtained from the fluroscence microscopy with arbitrary units were also compared with that of CASP programme in which the same fragments of DNA were automatically analysed in micrographs using highly developed software.

So far, a few studies have been carried out on the genotoxicity of pesticides, however, no study to the best of our knowledge has been carried out to determine the existence of DNA damage using advised or lower than those of advised doses of pesticides in human health via comet assay.

MATERIALS AND METHODS

When the negative effects of pesticides are evaluated on human carcinogenicity or for their hazardous effects on non-targeted organisms, generally the active substances are considered, however, in real time, pesticide users or non-targeted organisms are exposed to rather to a formulation which contains various additives and other active substances. In this study, the commercial pesticides were purchased from the local companies and used in the experiments; however, their names indicated by their active ingredients were given here instead of trade names.

Chemicals

Dimethoate (O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate), methly parathion (O,O-dimethyl O-P-nitrophenyl thiophosphate) and alphacypermethrin [(S)-alpha-cyano-3-phenoxybenzyl-(IR-3R) plus (R)-alpha-cyano-3-phenoxybenzyl-(1S-3S)-3 (2,2 dichlorovinyl)-2,2-dimethylcyclopropane carboxylate)] were purchased from the local pesticide markets. Other chemicals used in the comet assay were purchased from Sigma (www.sigma.com) unless otherwise stated. The chemicals used here were of analytical reagent grade quality from.

Blood samples and cell preparation

The peripheral blood samples (total 6 ml) were collected from an antecubital vein

into heparinised tubes (50 U/mol sodium heparin) stored at 2-4°C and kept in the dark to prevent further DNA damage, and were processed within 2 h. The donor was a 26 year-old non-smoking male not exposed to radiation or drugs and not has background of existence of diabetes mellitus, coronary artery disease, rheumatoid arthritis, malignancy, systemic or local infection, hypertension, acute-chronic liver diseases and anemia, which might negatively affect the condition of DNA.

Mononuclear leukocyte isolation for the comet assay was performed using the Histopaque 1077 (Sigma). An amount of 1 ml heparinised blood was carefully layered over 1 ml Histopaque and centrifuged for 35 min at 500*g* in 25°C. The interface band containing mononuclear leukocyte were washed with phosphate buffered saline (PBS) and then collected by 15 min centrifugation at 400*g*. The resulting pellets were resuspended in PBS and the cells were counted with an automatic cell counter (Abbott 3700, USA). Membrane integrity was assessed by means of trypan blue exclusion method. The cell concentrations were adjusted to approximately $2x10^5$/ml in the buffer. The cells were then suspended in a total volume of 1 ml containing 100 µl suspension ($2x10^4$ cells), varying microliter amounts of the pesticides, and PBS buffer to top up the volume to 1 ml. The pesticides were applied to blood cells with their applied and low doses, respectively, e.g. dimethoate (400-, 200 µg ml^{-1}); methyl parathion (360-, 180 µg ml^{-1}) and alphacypermethrin (100-, 50 µg ml^{-1}). The cells were incubated for 30 min at 37°C in an incubator together with the control samples. Each treatment consisted of 5 replicates.

Comet Assay

Slide preparation

After incubation period, the comet assay also known as single cell gel electrophoresis assay (SCGE) was performed as described by Singh et al (1988) with the following modifications: 10 µl of fresh mononuclear leukocyte cells suspension (around 20,000 cells) were mixed with 80 µl of 0.7% low-melting agarose in PBS at 37°C. Subsequently, 90 µl of mixture were layered onto a slide pre-coated with thin layers of 1% normal melting point (NMP) agarose and immediately covered with a coverslip. Slides were left for 5 min at 4°C to allow the agarose solidification. After removing the coverslips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris-HCl, pH 10 - 10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 1 h.

Electrophoresis

Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH, and 1 mmol/L Na_2EDTA, pH>13) at 4°C for unwinding (40 min) and then electrophoresed (25V/300 mA, 25 min). All the steps were carried out under minimal illumination. After electrophoresis, the slides were then neutralized (0.4 M/L Tris-HCL, pH 7.5) for 5 min.

Staining

The dried microscope slides were stained with ethidium bromide (2 µg/ml in distilled H_2O; 70 µl/slide), covered with a coverslip and analyzed using a fluorescence microscope (Olympus, Japan) at 400x magnification provided with epiflourescence and equipped with a rhodamine filter (excitation wavelength 546 nm; barrier 580 nm).

Scoring

Fifty cells were randomly scored by eye in each sample, on a scale of 0-4, based on fluorescence beyond the nucleus and were previously described by Kobayashi et al. (1995). The scale used was as follows: 0= no cometing; 1= comet <0.5 times the width of nucleus; 2= comet equals to width of nucleus; 3= comet greater than with of nucleus; 4= comet > twice the width of the nucleus. Scoring cells in this manner has been shown to be as accurate and precise as using computer image analysis. The individual scoring of the slides was blinded to any demographic or biochemical aspect of the blood sample. The visual score for each class was calculated by multiplication of the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score characterizing the degree of DNA damage in the entire examined samples was the sum of the scores in the five comet classes. Thus, the total visual score could range from 0 (all undamaged) to 400 (all maximally damaged) arbitrary units (AU). This visual scoring method had been suggested by Collins et al (1997). The comets were scored by doubled investigators. The above measurement was also performed using CASP analyzing programme to justify the above results and to obtain more parameters that cannot be possible

by visual scoring methods. Again, fifty cells from each of two slides were analysed from each treatment and the following parameters were obtained (Table 1).

Table 1. Some DNA parameters obtained from CASP programme to evaluate the DNA damage (Konca et al., 2003).

DNA damage parameters	Explanation and calculation of each parameter
L-Head	Length of Head DNA
L-Tail	Length of Tail DNA
L-Comet	Length of DNA (Whole)
DNA head (DNA-H)	sum of intensities of all points of the head
DNA tail (DNA-T)	sum of intensities of all points of the tail
Percent tailDNA (% DNA-T)	% DNA-T = 100 DNA-T/ (DNA-H + DNA-T).
Percent head DNA (% DNA-H)	% DNA-H = 100−% DNA-T.
Tail moment (TM)	the product of the tail length and percent tail DNA, TM = TL × (% DNA-T).
Olive tail moment (OTM)	the product of the distance (in x direction) between the center of gravity of the head (CGH) and the center of gravity of the tail (CGT) and percent tail DNA, OTM = (CGTx−CGHx)/% DNA-T

Structure of damaged DNA molecules have breaks inside thus allowing distruption of their supecoiled complex and liberate the breaks towards anode. Staining shows the DNA as "comets".

Statistical methods

The values were expressed as mean ± SE. The comparisons of parameters were performed using One-Way ANOVA test. The experiment was conducted with a randomized block design with 5 replications. A p value less than 0.05 were accepted as significant. Data were analyzed using SPSS® for Windows computing program (Version 11.0).

RESULTS

The cell viability was assessed with trypan blue dye excluding the live cells in each group. The viability was found more than 85%.

The aim of this study was to evaluate *in vitro* genotoxic effects of dimethoate, methyl parathion, alphacypermethrin in human peripheral lymphocytes with the comet assay. The results showed that dimethoate, methyl parathion, alphacypermethrin significantly increased DNA damages (Fig 1). The elaborated examination on DNA fragments also showed that all parameters including tail length, comet length, % T-DNA, TM and OTM values significantly increased, p <0.05 (Table 2). The advised doses or lower than those of advised doses of each respected pesticide caused an increased significant DNA damage on peripheral lymphocytes although that the lower doses of each pesticide has less damaging effect. The amount of DNA breakage in a cell in the comet assay, which is the migration of genetic material from DNA head towards anode, is used to measure of the condition of DNA (Fig 2). From the results of experiments, TM and OTM values of each treatment group significantly increased above the control values both at advised and lower doses. However, methyl parathion and alphacypermethrin, both of which are highly toxic chemicals (LD_{50s} 3 and 57 µg ml^{-1}, respectively) caused intensive DNA damages when TM and OTM values were compared with that of control group at both concentration levels (Table 2). Dimethoate, on the other hand, caused significant DNA damages to a lesser extent when compared to other chemicals, however, lower doses of dimethoate was also toxic to peripheral lymphocytes (Table 2).

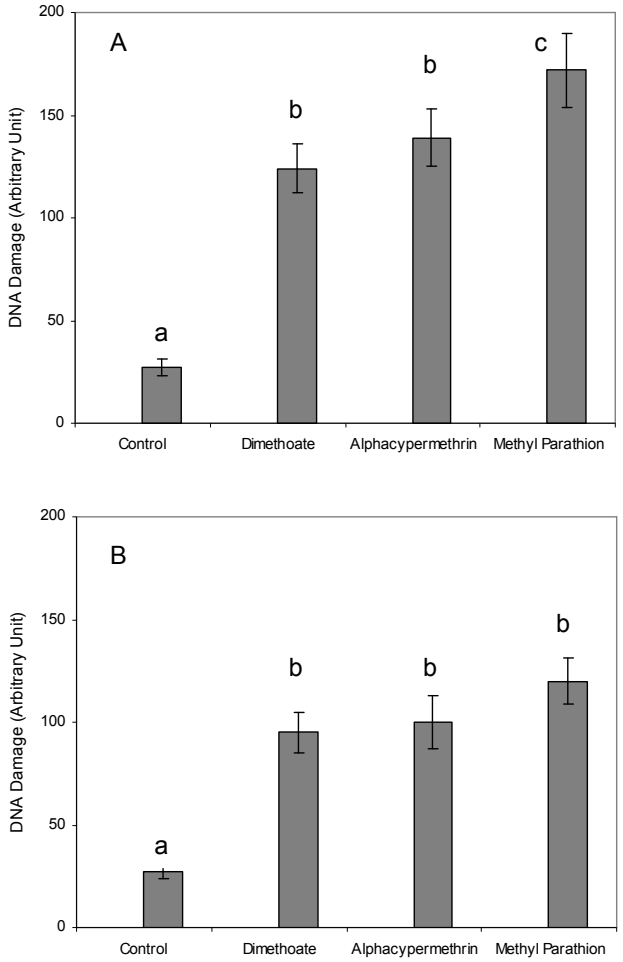

Fig 1. Effects of pesticides on peripheral monouclear DNA damage levels assessed by Single Cell Gel Electrophoresis. A-The effect of advised dose of each chemical group (Dimethoate, 400-; Methyl parathion, 360-; Alphacypermethrin, 100 µg ml^{-1}). B-Effect of low doses (Dimethoate, 200-; Methyl parathion, 180-; Alphacypermethrin, 50 µg ml^{-1}). Letters different from each other show significant differences. Values were expressed as mean ± SE ($p < 0.05$).

Table 2. Grades of DNA damage and comet assay parameters on peripeheral lymphocytes treated with dimethoate, alphacypermethrin and methyl parathion.

Characterisitcs of pesticides			Parameters							
Pesticides	*LD_{50} mg kg^{-1}	Advised dose (µg ml^{-1})	L Head	L Tail	L Comet	% H DNA	% T DNA	T DNA/ H DNA	TM	OTM
Control	-	-	44 [a 1]	4[a]	48 [a]	99 [a]	1 [a]	0.01	0.01 [a]	0.01 [a]
Dimethoate	387	400	63 [b]	27 [b]	89 [b]	86 [b]	14 [b]	0.16	6.05 [b]	5.22 [b]
Methyl parathion	3	360	53 [b]	35 [b]	88 [b]	79 [b]	21 [b]	0.27	9.09 [b]	8.04 [b]
Alphacypermethrin	57	100	51 [b]	28 [b]	79 [b]	82 [b]	18 [b]	0.22	9.90 [b]	7.65 [b]
		Low dose (µg ml^{-1}) (µg ml^{-1})								
Dimethoate		200	52 [b]	21 [b]	73 [b]	87 [b]	13 [b]	0.15	4.10 [c]	3.61 [c]
Methyl parathion		180	52 [b]	29 [b]	81 [b]	83 [b]	17 [b]	0.20	6.57 [b]	5.57 [b]
Alphacypermethrin		50	51 [b]	28 [b]	79 [b]	86 [b]	14 [b]	0.17	6.51 [b]	5.58 [b]

* Acute oral effect on rats.

[1] Parameters with the same letters in the columns are *not significantly different* from each other at 0.05 level.

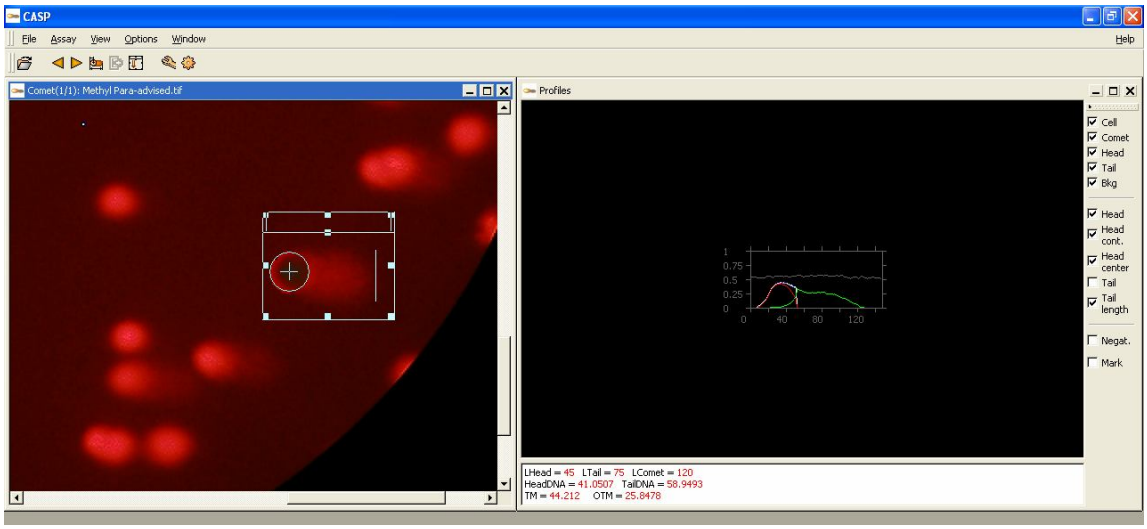

Fig 2. DNA micrograpgs analysed with CASP programme; in the left hand window, DNA fragments with a head and a tail and in the right hand window, intensity profiles are plotted with the results of selected measurements.

DISSCUSSION and CONCLUSION

Tail length, comet length, % T-DNA, TM and OTM values were significantly increased at all treatments peripheral lymphocytes with dimethoate, methyl parathion and alphacypermethrin treatments. By and large, the genotoxic effects of the tested pesticides appeared to be better reflected by TM and OTM values. The genotoxic effects of pesticides on higher cells caused an increased DNA damage. For example, *in vitro* studies on human and Chinese hamster cell lines showed positive evidence for genotoxicity when treated with different pesticides (Moretti et al. 2002). Results obtained from many *in vitro* studies showed that different pesticides were cabable of inducing choromosomal aberrations (Au et al. 1999) and increase in the number of miconuclei in peripheral blood lymphocytes (Falck et al. 1999) and sister chromatid exchanges (Rupa et al. 1989). In the last decade, a few good reports with the comet assay which was applied as a rapid and sensitive method to evaluate the genotoxic hazard of pesticides were reported. For example, Garaj-Vrhovac and Zeljezic (2000) and Undeger and Basaran (2002 & 2005) found that the DNA damage of peripheral lymphocytes significantly increased with the increase in concentration of pesticides. However, the information about the genotoxic potential of some of pesticides is limited and inconsistent. Single-cell gel electrophoresis has become a sensitive and rapid method for the detection of DNA damage at the individual cell level. It is a useful assay for simultaneous comparison of effects of different chemicals in somatic cells.

In this work, genotoxic effects of pesticides were remarkably caused extensive DNA damages at advised and lower than those of advised doses at all treatments. Methyl parathion and alphacypermethrin, highly toxic chemicals, caused a significant DNA damages even at low concentrations. The comparison of DNA damages as arbitrary units (Fig. 1) and detailed data in fragments of DNA were conclusive and the detailed examination of DNA revealed that the other parts of DNA such as tail and tail area were badly affected as reflected by the TM and OTM values.

There are some studies reporting DNA damage with several pesticides with *in vitro* alkaline comet assay, however, those workers tested either very low doses or not practically advised doses on higher cells (Undeger and Basaran, 2005). Although their findings are quite significant and they determined DNA damages in peripheral lymphocytes, here, with advised doses and lower than those of advised doses of each pesticide was tested on higher cells. The results were quite remarkable that the real time doses could cause extensive DNA damages even with their lower doses, which could also be cabable of causing same extent of DNA damages. The interesting side of these findings is that the DNA damages could be caused by even environmentally friendly chemicals that their mode of action in the metabolism is not dangerous as other chemicals.

The results presented in this paper as well as the results of other authors pointed out

the capability of pesticides in causing genome damage.

In conclusion, the work presented here provided evidence for the genotoxicity of pesticides and confirmed the usefulness of the comet assay in higher cells. Due to the simplicity, reproducibility and rapidity of this technique as well as the possibility of detection of DNA damage in the individual cell by this technique, it is widely used in genotoxicology and mutagenecity studies, therefore, we suggest the use of comet assay for the determination of single- and double-DNA strand breaks as well as alkali-labile sites in routine genotoxicity tests in organisms exposed to pesticides whether it is highly toxic or friendly. All cell types can be examined with this assay as long as they can be individually recognized.

Although the results here were obtained from the study of blood cells *in vitro* conditions, it would be very useful to determine the effect of those pesticides *in vivo*. Here, we demonstrated that the living cells could undergo a series of negative changes when exposed to friendly chemicals.

The future work with these chemicals is to investigate their damaging effects with more concentrations using other parameters such as antioxidant enzymes and total antioxidant capacity and MDA (malondialdehyde) levels on non-targeted orgaisms as well as targeted organisms.

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REFERENCES

Au, W.W, Sierra-Torres, C.H., Cajas-Salazar, N., Shipp, B.K., Legator, M.S. 1999. Cytogenetic effects from exposure to mixed pesticides and the influence from genetic susceptibility. *Environmental Health Perspectives* 107:501–505.

Barthel, E. 1981. Increased risk of lung cancer in pesticide-exposed male agricultural workers. *Journal of Toxicolology and Environmental Health* 8:1027–1040.

Blair, A., Zahm, S.H. 1995. Agricultural exposures and cancer. *Environmental Health Perspectives* 103:205–208.

Cavas, T., Konen, S. 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. *Mutagenesis* 22:263–268.

Collins, A., Dussinska, M., Franklin, M. 1997. Comet assay in human biomonitoring studies: reliability validation and application, *Environmental and Molecular Mutagenesis 30*: 439-462.

Collins, A.R. 2002. The comet assay. Principles, applications and limitations. *Methods in Molecular Biology* 203:163–177.

Das, P., John, G. 1999. Induction of sister chromatid exchanges and chromosome aberrations *in vivo* in Etroplus suratensis (Bloch) following exposure to organophosphorus pesticides. *Toxicology Letters* 4:111–116.

Dikilitas, M., Kocyigit, A. ve Yigit, F. 2009. A molecular-based fast method to determine the extent of DNA damages in higher plants and fungi. *African Journal of Biotechnology*, 8 (14): 3118-3127.

Dimitrov, B.D., Gadeva, P.G., Benova, D.K., Bineva, M.V. 2006. Comparative genotoxicity of the herbicides roundup, stomp and reglone in plant and mammalian test systems. *Mutagenesis* 21:375–382.

Ecobichon, D.J. 2001. Toxic effects of pesticides. In: Klaassen CD (ed). Casarett and Doull's toxicology. The basic science of poisons. McGraw-Hill, New York, pp 763–810.

Falck, G.C.M., Hirvonen, A., Scarpato, R., Saarikoski, S.T., Migliore, L., Norppa, H. 1999. Micronuclei in blood lymphocytes and genetic polymorphism for GSTM1, GSTT1 and NAT2 in pesticide exposed greenhouse workers. *Mutation Research* 441:225–237.

Garaj-Vrhovac, V., Zeljezic, D. 2000. Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay. Pesticide genotoxicity revealed by comet assay. *Mutation Research* 469:279–285.

Garrett, N.E., Stack, H.F., Waters, M.D. 1986. Evaluation of the genetic activity profiles of 65 pesticides. *Mutation Research* 168: 301–325.

Gichner, T., Znidar, I., Wagner, E.D. ve Plewa, M.J. 2009. The use of higher plants in the comet assay. In: The Comet Assay in Toxicology Edited by Alok Dhawan and Diana Anderson. Royal Society of Chemistry, p: 98-119.

Hayes, W.J., Laws, E.R. 1990. Handbook of Pesticide Toxicology, Vol. 3, Classes of Pesticides. Academic Press, Inc., NY.

Howard, P.H. 1989. Handbook of Environmental Fate and Exposure Data for Organic Chemicals, Vol. III: Pesticides. Lewis Publishers, Chelsea, MI.

Kobayashi, H., Sugiyama, C., Morikawa, Y., Hayashi, M.,. Sofuni, T. A. 1995. Comparison between manual and microscopic analysis and computerized image analysis in single cell electrophoresis assay. *MMS Communication* 3:103-115.

Kocyigit, A., Keles, H., Selek, S., Guzel, S., Celik, H. ve Erel, O. 2005. Increased DNA damage and oxidative stress in patients with *Cutaneous leishmaniasis*. *Mutation Research*, 585:71-78.

Konca, K., Lankoff, A., Banasik, A., Lisowska, H., Kuszewski, T., Gozdz S, Koza, Z. ve Wojcik, A. 2003. A cross-platform public domain PC image-analysis program for the comet assay. *Mutation Research* 534: 15-20.

Lebailby, P., Vigreux, C., Lechevrel, C., Ledemeney, D., Godard, T., Sicheb, F., LeTalaer, J. Y. Henry-Amar, M, Gauduchon P. 1998. DNA Damage in Mononuclear Leukocytes of Farmers Measured Using the Alkaline Comet Assay: Discussion of Critical Parameters and Evaluation of Seasonal Variations in Relation to Pesticide Exposure. *Cancer Epidemiology, Biomarkers & Prevention* 7: 917-927.

Lucero, L., Pastor, S., Suarez, S., Durban, R., Gomez, C., Parron, T., Creus, A., Marcos, R. 2000. Cytogenetic biomonitoring of Spanish greenhouse workers exposed to pesticides: micronuclei analysis in peripheral blood lymphocytes and buccal epithelial cells. *Mutation Research* 464:255–262.

Meister, R.T. 1992. Farm Chemicals Handbook '92. Meister Publishing Company, Willoughby, OH.

Moretti, M., Marcarelli, M., Villarini, M., Fatigoni, C., Scassellati-Sforzolini, G., Pasquini, R. 2002. *In vitro* testing for genotoxicity of the herbicide terbutryn:cytogenetic and primary DNA damage. *Toxicology in Vitro* 16:81–88.

Ostling, O., Johanson, K.J. 1984. Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communications* 30: 291-298.

Rupa, D.S., Reddy, P.P., Reddi, O.S. 1989. Analysis of sister-chromatid exchanges, cell kinetics and mitotic index in lymphocytes of smoking pesticide sprayers. *Mutation Research* 223:253–258.

Singh, N.P. McCoy, M.T., Tice, R.R., Schneider, E.L. 1988. A simple technique for quantification of low levels of DNA damage in individual cells. *Experimental Cell. Research* 175: 184–191.

Sinha, S.P. 1989. Genotoxicity of pesticides. *Perspectives in Cytology and Genetics* 6:749–753.

Undeger, U., Basaran, N. 2002. Assessment of DNA damage in workers occupationally exposed to pesticide mixtures by the alkaline comet assay. *Archives Toxicology* 76:430–436.

Undeger, U., Basaran, N. 2005. Effects of pesticides on human peripheral lymphocytes in vitro: induction of DNA damage. *Archives Toxicology* 79: 169-176.

Undeger, U., Institoris, L., Siroki, O., Nehez, M., Desi, I. 2000. Simultaneous geno- and immunotoxicological invesitgations for early detection of organophosphate toxicity in rats. *Ecotoxicological Environmental Safety* 45:43–48.

Velazquez, A., Xamena, N., Creus, A., Marcos, R. 1990. Mutagenic evaluation of the organophosphorus insecticides methyl parathion and triazophos in *Drosophila melanogaster*. *Journal of Toxicological Environmental Health* 31:313–325.

Viel, J.F., Chalier, B. 1995. Bladder cancer among French farmers: does exposure to pesticides in vineyards play a part? *Occupational and Environmental Medicine* 52:587–592.

Weisenburger, D.D. 1993. Human health effects of agrichemical use. *Human Pathology* 24, 571–576.

WHO, 1988. The WHO recommended classification of pesticides by hazard and guidelines to the classification 1988–1989. World Health Organisation, Geneva.

WHO, 1993. Environmental Health Criteria 145. Methyl parathion. World Health Organization, Geneva. 1993.