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Evaluation of genotoxic potentials in agricultural workers exposed to pesticides

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Abstract
Introduction: Regarding pesticide exposure, many reports dealing with Sister chromatid exchange (De Ferrari *et al.*, 1991; Garaj-Vrhovac and Zeljezic, 2001; Shaham *et al.*, 2001) and Single cell gel electrophoresis (Garaj-Vrhovac and Zeljezic, 2000; Zeljezic and Garaj-Vrhovac, 2001) found significant increases in these biomarkers, providing suggestive evidence of genotoxic effects induced by pesticides. The present study aimed to assess the genotoxic effects of the pesticides on occupationally exposed agricultural workers at paddy cultivation.
Methods: Sample size of the present study includes 30 agricultural workers exposed to pesticides in Kakinada, East Godavari District, Andhra Pradesh, India and 20 healthy normal individuals of the same age and sex from the same region who are not exposed to pesticides as the controls, with age above 20 during the period March 2015 to September 2015. 5ml of blood sample collected from both study and control groups for genotoxicity study, by using comet assay and micronuclei assay.
Results: The comet tail length mean value of exposed people to pesticides was high when compared with the nonexposed people to pesticides. Significant increase in micronuclei and binucleated cells was observed in people exposed to pesticides compared to nonexposed controls, where as the mean frequency of single nucleated cells in nonexposed controls was more when compared with the exposed individuals.
Conclusions: The present study correlates with the findings of earlier studies carried out in India and other countries. It emphasizes the workers exposed to pesticides presented more genomic damages than non exposed people either evaluated by comet assay or micronuclei assay.

Keywords: Agricultural workers, Genotoxicity, Pesticides, Micronuclei, DNA damage.

Introduction
Synthetic pesticides emerge between 1930 and 1940 as a result of research aimed at developing chemical weapons that were originally tested in insects. One of the first compounds, dichlorodiphenyltrichloroethane (DDT) was synthesized by Zeidler in 1874, and its insecticidal properties were described by Paul Müller in 1939. DDT was first used during World War II to protect American soldiers carried diseases by vector and was marketed in the U.S in 1945 (WHO, 1990).
Pesticides are extensively used all over the world to increase food production and control vector-borne diseases and in recent years their use was increased dramatically. Unfortunately, large amounts of these chemicals are released into the environment and many of them affect non-target organisms, being a potential hazard to human health. Fifty-six pesticides have been classified as carcinogenic to laboratory animals by the IARC (IRAC, 2003). Meta-analyses showed that pesticide-exposed farmers are at risk for specific tumors, including leukemia, (Zahm *et al*., 1997; Zahm *et al*., 1998; Daniels *et al*., 1997) [60, 61, 13]; Non-Hodgkin's lymphoma, (Zheng *et al*.,2001) [63]; soft tissue sarcoma, (Kogevinas *et al*., 1995) [30]; Parkinson disease, (Wood Ward, 2001) [58]; multiple myeloma, (Viel *et al*., 1998) [57]; stomach and prostate malignancies (Bolognesi, 2003) [6]. These effects are not always related to immediate and apparent injuries, but can take even years to manifest (Zuniga, 2006) [64].
Alkylating abilities of the pesticide chemicals induce nicks in DNA (Velazquez *et al*., 1986) [55] and thus affect DNA replicating ability and its ability to carry information (Islas-Gonzalez *et al*., 2005) [25]. DNA damage together with cellular response can establish genomic instability through multiple pathways (Gontijo *et al*., 2001) [26] and can be considered as an effective strategy for risk assessment. Individuals occupationally exposed to pesticides

have great genotoxic risk and assessment of this risk in exposed subjects can be used as fairly reliable biomarker of early biological alterations (Garaj-Vrhovac *et al*., 2001) [22]. Biomarkers frequently used to assess genotoxic effects of pesticides include micronuclei formation and comet assay.

As most occupational and environmental exposures are exposure to mixtures of pesticides, the genotoxic potential evaluated on single compounds could not be extrapolated to humans. Hence, the genotoxicological assessment in human populations is a useful tool to estimate the genetic risk from an integrated exposure to complex mixtures of pesticide. Several cytogenetic assays have been used to evaluate the potential genotoxicity of pesticide exposures in occupationally exposed populations. However, there are reports on positive genotoxic effects in populations exposed to pesticides (Bolognesi *et al*., 1993; Kourakis *et al*., 1996; Antonucci *et al*., 2000) [5, 31, 2] as well as negative findings (Scarpato *et al*., 1996; Lucero *et al*., 2000) [50, 35].

Regarding pesticide exposure, many reports dealing with SCE (De Ferrari *et al.*, 1991; Garaj-Vrhovac and Zeljezic, 2001; Shaham *et al.*, 2001) [15, 22, 52] and SCGE (Garaj-Vrhovac and Zeljezic, 2000; Zeljezic and Garaj-Vrhovac, 2001) [23, 22] found significant increases in these biomarkers, providing suggestive evidence of genotoxic effects induced by pesticides. The present study aimed to assess the genotoxic effects of the pesticides on occupationally exposed agricultural workers at paddy cultivation.

Methods

Sample size of the present study includes 30 agricultural workers exposed to pesticides in Kakinada, East Godavari District, Andhra Pradesh, India and 20 healthy normal individuals of the same age and sex from the same region who are not exposed to pesticides as the controls, with age above 20 during the period March 2015 to September 2015. 5ml of blood sample collected from both study and control groups for genotoxicity study.

Comet Assay

In vitro test was performed according to the method of Singh *et al*., 1988 with small modifications. 1×10^6 lymphocytes were suspended in 0.5% low melting point agarose and sandwiched between a layer of 0.6% normal melting point agarose and a top layer of 0.5% low melting point agarose on fully frosted slides. During polymerization of each gel layer the slides were kept on ice. After solidification of the 0.6% agarose layer the slides were immersed in lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and 10% DMSO) at 4°C. After 1 hour slides were placed in electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 10) for 20 min at room temperature to allow for DNA unwinding. Electrophoresis was conducted in a horizontal electrophoresis platform in fresh, chilled electrophoresis buffer for 20 min at 300 mA and 19 V. The slides were neutralized with Tris-HCl buffer, pH 7.5, three times for 5 min and stained with 10% ethidium bromide for 10 min. Each slide was analyzed using a fluorescence microscope equipped with a 515-560 nm excitation filter. For each subject 50 cells were analyzed with an automatic digital analysis system, determining tail length.

Micronuclei Assay

The *in vitro* test was performed according to the method of Fenech and Morley, 1986 [19] with small modifications. Add 0.5 ml of heparinized whole blood in 5 ml of RPMI 1640 supplemented with 20% fetal bovine serum, 50 ml L-glutamine, antibiotics, and 1.0% phytohemagglutinin. Incubated the tubes at 37 ºC for 44 hours, later add 37.5 µl of cytochalasin-B to each tube. At 72 hours, make a gentle fixation of methanol/acetic acid (3:1). Cell suspensions are dropped from each tube onto five clean and cold slides from a 2-3 cm distance and a slide inclination of 45º.Slides are allowed to air dry and incubated at 60 ºC overnight and stained with Giemsa stain. The number of multinucleate cells and number of bi nucleate cells are counted to calculate the mean percent (%) of cells at multinucleate stage.

Results

Table 1: Mean comet tail length (µm) in exposed and nonexposed people to pesticides

Groups Tail length (µm)	
	Mean± Standard Deviation
Exposed(30)	
Males(20)	13.29 ± 1.54
Females(10)	11.12 ± 0.95
Total	12.57 ± 1.71
Non-exposed(20)	
Males(10)	3.04 ± 1.14
Females(10)	4.36 ± 1.2
Total	3.70 ± 1.34

Table 1 represents the mean values of comet tail length in exposed and non exposed people to pesticides. The mean values of males and females in exposed people to pesticides were 13.29 and 11.12 respectively. The mean value of nonexposed males to pesticides was 3.04 and females was 4.36. The total mean value of exposed people to pesticides was 12.5 where as the total mean value of nonexposed people to pesticides was 3.70. From the above table it was inferred, that the comet tail length mean value of exposed people to pesticides was high when compared with the nonexposed people to pesticides.

Table 2: Mean frequency of micronuclei in exposed and non exposed people to pesticides.

Groups		**Parameters**	
	Micronuclei (±)SD	Binucleated (±) SD	Single nucleated (±) SD
Exposed			
Males(20)	2.75 ± 1.44	12.55 ± 2.41	84.7 ± 2.83
Females(10)	3.6 ± 1.42	14.05 ± 1.82	82.35 ± 2.3
Total	3.1 ± 1.4	13.3 ± 2.27	83.52 ± 2.83
Non-exposed			
Males(10)	0	4.7 ± 1.95	95.30 ± 1.95
Females(10)	0	5.1 ± 2.21	94.9 ± 2.21
Total	0	4.9 ± 2.09	95.1 ± 2.09

Table 2 represents the mean frequency of micronuclei in exposed and nonexposed people to pesticides. The mean frequency of micronuclei in exposed males and females to pesticides were 2.75 and 3.6 respectively and micronuclei were completely absent in nonexposed people to pesticides.

The mean frequencies of binucleated cells in exposed males and females to pesticides were 12.55 and 14.05 respectively, where as the mean frequency of nonexposed males was 4.7 and females was 5.1.

The mean frequencies of single nucleated cells in exposed males and females to pesticides were 84.7 and 82.35 respectively, where as the mean frequency of nonexposed males to pesticides was 95.30 and females was 94.9.

The total mean frequencies of micronuclei and binucleated cells of people exposed to pesticides were 3.1 and 13.3 respectively, where as the total mean frequency of single nucleated cells exposed to pesticides was 83.52.

The total mean frequencies of binucleated cells and single nucleated cells in people nonexposed to pesticides were 4.9 and 95.1 respectively, where as the micronuclei were completely absent.

It was evident from the above table, significant increase in micronuclei and binucleated cells was observed in people exposed to pesticides compared to nonexposed controls, whereas the mean frequency of single nucleated cells in nonexposed controls was more when compared with the exposed individuals.

Discussion

Base line genetic damage is influenced by various intrinsic and extrinsic factors, but it is not yet clear how an individual's inborn genetic constitution may influence yield of such damage. For this reason, assessment of level of DNA damage in 30 farmers occupationally exposed to pesticides along with 20 controls nonexposed to pesticides was done.

As with the results obtained in present work, people exposed to pesticides presented more genomic damages than non-exposed people, evaluated by the comet assay. The present work correlated with works of (Yoder *et al*., 1973; Dulout *et al.*, 1985; Paldy *et al.*, 1987; Rita *et al.*, 1987; Nehez *et al*., 1988; Rupa *et al.*, 1989c, 1991; De Ferrari *et al*., 1991; Kourakis *et al*., 1992; Bolognesi *et al.*, 1993; Carbonell *et al.*, 1993; Lander and Ronne, 1995; Falck *et al.*, 1999; Munnia *et al.*, 1999; Antonucci and Colus, 2000; Gomez-Arroyo *et al.*, 2000; Shaham *et al.*, 2001; Garaj-Vrhovac and Zeljezic 2002; Grover *et al*., 2003; Simoniello *et al*. 2008; Agopian *et al*. 2009; Bortoli *et al*. 2009; Paiva *et al*. 2011; Benedetti *et al*. 2013;) [59, 16, 42, 47, 39, 48, 15, 32, 5, 9, 34, 18, 38, 2, 24, 52, 21, 27, 53, 1, 7, 41, 3]. However, results obtained by some researchers using various cytogenetic assays with populations exposed to pesticides elucidated negative results unlike the present study (Crossen and Morgan, 1978; Carbonell *et al.*, 1990; Gomez-Arroyo *et al.*, 1992; Hoyos *et al.*, 1996; Scarpato *et al.*, 1996; Davies *et al.*, 1998; Venegas *et al.*, 1998; D'Arce and Colus, 2000; Lucero *et al.*, 2000; Pastor *et al.*, 2001a,b; Zeljezic and Garaj-Vrhovac, 2001) [8, 25, 28, 50, 14, 56, 11, 35, 44, 45, 22].

Some contradictory results regarding genetic monitoring of populations exposed to pesticides are due to multiple exposures, different types of pesticides, or the heterogeneous composition of the population (Scarpato *et al*., 1996a, b) [50, 51]. The use of pesticides has brought a number of consequences for the environment and for the health of farmers. These consequences are conditioned by factors that are intrinsically related, such as the in appropriate use of these substances, the high toxicity of certain products, the lack of utilization of protection equipment, and the insecurity of surveillance (Oliveira-Silva *et al*. 2001) [40].

The present study shows higher number of micronuclei or binucleated cells in people exposed to peticides than nonexposed people. The positive result also observed in the studies of (Carbonell *et al*., 1993; da Silva Augusto *et al.*, 1997; Falck *et al*., 1999; Bolognesi and Morasso 2000; Gomez-Arroyo *et al*., 2000; Antonucci and De Syllos, 2000; Garaj-Vrhorac and Zeljezic, 2000, 2001; Figgs *et al.*, 2000; Shaham *et al.*, 2001; Pastor *et al*., 2003; Ergene *et al*. 2007; Costa *et al*. 2007; Martínez-Valenzuela *et al*. 2009; Remor *et al*. 2009; Kvito *et al*. 2012) [9, 12, 18, 4, 24, 2, 23, 22, 20, 52, 43, 17, 10, 36, 46, 33]. The negative results obtained in studies of (Hoyos *et al*., 1996; Scarpato *et al*., 1996b; Davies *et al.*, 1998; Venegas *et al.*, 1998; Lander *et al.*, 2000; Gregorio d'Arce and Colous, 2000; Lucero *et al*., 2000; Pastor *et al*., 2001a, b, 2002) [28, 51, 14, 56, 11, 35, 44, 45] which does not correlates with the present study.

The differences between results obtained by biological monitoring of groups exposed to pesticides may reflect different exposure conditions, such as magnitude of exposure, protective measures, specific genotoxic potential, type of culture, environmental factors, endogenous factors, formulation, and potential for absorption of the pesticide, and laboratory technique used. For these reasons, a genotoxicity study conducted in a specific occupational risk condition cannot be extrapolated by other situations that occur in genetic and occupational risks involved (Bolognesi and Morasso, 2000; Sailaja *et al*., 2006) [4, 49].

This paper presented an association between pesticide exposure and an increase in either DNA damage or in the number of micronuclei. So, our findings revealed that pesticides in long-term exposure could act as mutagenic agents. But unfortunately, as the farmers were exposed to distinct products, it is not possible to determine the effect of a specific compound on the genome of such workers.

Still now there is no clear conclusion regarding the available biomonitoring studies of genotoxicity following occupational exposure to pesticides. One factor, which appreciably influenced this conclusion, is the extreme variation in the biomonitoring indices frequency in the control populations examined in the selected studies. In this respect, it is very difficult to infer causality for the small magnitude of responses seen in the positive studies. Furthermore, it is concluded that factors affecting variance in genotoxicity end points are not adequately understood and there is a clear need for more data on the background variability in the general population.

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