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The Investigation of Clastogenic Effects of α-Endosulfan and β-Endosulfan on Spraque–Dawley Rats by Micronucleus Test Method

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Abstract
My study's importance is the investigation of the clastogenic effects of endosulfan isomers (α and β endosulfan), on Spraque - Dawley male albino rats by the micronucleus test system. One sixth and 1 / 12 of intraperitonal Lethal Dose$_{50}$ for α - endosulfan (12 mg / kg and 6 mg / kg), and 1 / 12 and 1 / 24 of intraperitonal Lethal Dose$_{50}$ for β - endosulfan (20 mg / kg and 10 mg / kg) were used in the study. The rats intraperitoneally injected with insecticides were killed by cervical dislocation after 72 h of the last application. The preparations were made from the smears of the femoral bone marrow and then examined under the light microscope. The erythrocyte count in the preparations was performed by whole-region scanning in each preparation; 1000 erythrocytes were counted in every preparation. Polychromatic erythrocytes, micronucleated polychromatic erythrocytes and normochromatic erythrocytes were counted in the control and treatment groups. In the groups given α - endosulfan and β - endosulfan, a statistically significant increase was found in the numbers of polychromatic erythrocytes and micronucleated polychromatic erythrocytes. Also, a significant increase was observed in the number of micronuclei. An increase in the number of micronucleated polychromatic erythrocytes is an indication of clastogenic effect, which has a negative influence on both individuals and their future generations. In conclusion, the present study showed that α and β isomers of endosulfan exert a clastogenic effect in the bone marrow leading to toxicity in rats.
Keywords: Bone marrow, Endosulfan isomers, Polychromatic erythrocytes, Micronucleated polychromatic erythrocytes, Normochromatic erythrocytes.

1. Introduction

The production of crops from agricultural areas has been insufficient to meet the needs of the growing world population. Therefore, besides improving the agricultural techniques, various chemicals have been used against microorganisms, insects, and weeds that damage the plants and their products. Although, these chemicals called pesticides have short - term benefits, they cause environmental pollution if they stay in water, and on soil and food for a long time. Moreover, these chemicals lead to serious health problems by entering the human body through the food chain (Buhunya & Pati 1988; Aaron et al. 1989).

Of many pesticides used nowadays, one of the the most important pesticides is "endosulfan." Generally pesticides have varied effects, especially clastogenic, teratogenic, and mutagenic in the living beings (Aaron et al. 1989; Boereboomet et al. 1998). A clastogenic effect is the effect causing chromosomal breakage. An average lethal dose of a toxic substance having a clastogenic effect is called Lethal Dose$_{50}$ (LD$_{50}$) (Kilbey et al. 1984). LD$_{50}$ value is used to show the acute toxicity of a substance. For pesticide application in rats, one third or one sixth of intraperitoneal (i.p.) LD$_{50}$ can be given daily, but if these doses have a lethal effect, then 1 / 12 or 1 / 24 of LD$_{50}$ can be applied (Hayes 1984; Kilbey et al. 1984; Grover & Malhi 1985). The clastogenic effect is determined using the micronucleus test (Dzwonkowska et al. 1986; McGregor 1998). This method determines the clastogenic effect of the used substance in the bone marrow cells. Using this method, polychromatic erythrocytes (PCE), micronucleated polychromatic erythrocytes (MNPCE), and normochromatic erythrocytes (NCE) in the bone marrow cells are investigated, and then the clastogenic effect of the used substance is determined. The increase in the number of PCE and MNPCE is an indicator of the clastogenic effect (Dzwonkowska et al. 1986; McGregor 1998; Dalsenter et al. 2003; Frederick & Myna 2003).

The effects of different chemicals on different living beings have been studied using the micronucleus test by various researchers. A study found that deltamethrin causes toxicity in mice (Özkan & Üstüner 2012). Another study on the mutagenic effects of chemical agents to which the workers in the printing industry are exposed showed that the number of MNPCE in the oral mucosa cells increased in the workers than in the the normal population (Yıldırım & Yıldırım 2011). It has been reported that methylcholantrene increased the number of MNPCE and decreased the mitotic activity in the mice by exerting a cytotoxic effect (Aksu et al. 2013). A study on the effects of environmental pollutants on plants demonstrated that the environmental pollutants caused numerical and structural chromosomal aberrations in the meristematic cells in the roots of plants with cytologic damages (Akyıl & Özkara 2015). Another study explored the effect of different doses of the application of fenpiroksimat on the *Allium cepa* seeds and found that the number of MNPCE and chromosomal aberrations increased in the treatment group significantly (Karakuşlu et al. 2015).

This study investigated clastogenic effects of α - endosulfan and β - endosulfan in the Spraque – Dawley male albino rats using the micronucleus test method. The clastogenic effects of the isomers of endosulfan in the bone marrow were identified by the changes in the number of PCE and MNPCE. Since a similar effect may also occur in human beings, the issue of pesticide use should be paid special attenton. It is believed that this study might serve as a base for future studies on this subject

2. Materials and methods

2.1. Animals

Three months old Spraque – Dawley male albino rats weighing 200 ± 20 g were used in this study. The rats were fed daily under the standard conditions (room temprature 20 ± 1 ºC, relative humudity 50 ± 10 % 12 / 12 h light – dark cycle) without food and water restriction.

2.2. Pesticides and features

The isomers α - endosulfan (CAS; Chemical Abstracts Service: 959-98-8), 96 % pure, and β - endosulfan (CAS; Chemical Abstracts Service: 33213-65-9), 98.9 % pure, were obtained from Sigma – Aldrich Laborchemikalien Gmbh D – 918, Taufkirchen Germany.

2.3. Pesticide doses and their preparation

A total of 25 rats were divided into 5 groups (n = 5). The rats in the first group, termed as the control group, were given 1 mL of dimethyl sulfoxide (DMSO) intraperitoneally. The rats in the second group were given 12 mg / kg, one sixth of LD_{50} (76 mg / kg) (Macfarlane 1999), of α - endosulfan intraperitoneally on the right side of the abdomen under the skin. The rats in the third group were given 6 mg / kg, 1 / 12 of LD_{50}, of α -endosulfan intraperitoneally. The rats in the fourth group were given 20 mg / kg, 1 / 12 of LD_{50} (240 mg / kg) (Macfarlane, 1999), of β - endosulfan intraperitoneally on the same area again. The rats in the fifth group were given 10 mg / kg, 1 / 24 of LD_{50} of β - endosulfan intraperitoneally. Every dose was dissolved in 1 mL of DMSO and applied for five consecutive days.

2.4. Preparations for micronucleus test method

The animals were killed by cervical dislocation, and the femurs were removed. The femurs were cleaned of muscles and used for the micronucleus test. Bone marrow preparations were prepared by the method improved by Schmid (Schmid 1985). Each femur was cut from the ends, and the bone marrow was transferred with an injector into the centrifuge tube containing 3 mL of fetal bovine serum. The tubes were centrifuged at 2000 g for 5 min. After removing the supernatant, the rest was suspended by adding a drop of fetal bovine serum and spread on the slides. The slides were dried in air for 10 min and fixed in methanol. They were painted with 0.25 % May – Grünwald paint for 5 min and washed with pure water. Next, they were painted with 0.125 % May – Grünwald paint for 5 min and washed with pure water. Finally, they were painted with 20 % Giemsa paint for 30 min, washed with pure water, and dried in air. One day after painting, the preparations were sealed with synthetic resin and examined under a light microscope. In every preparation, 1000 PCE were counted. MNPCEs were also counted and their percentage was determined. Moreover, 1000 erythrocytes (PCE + NCE) were counted from every preparation, and the ratios of PCE / NCE were detected.

2.5. Statistical analysis

Data in the groups were statistically analyzed using the SPSS (9.0.0) program. The data were evaluated using the Mann – Whitney U test. P value less than 0.05 was considered statistically significant.

3. Results

Six rats died in the experiment in 5 days. Including three rats in the second group and one rat in every other groups. The micronucleus test was performed on live rats.

3.1. Micronucleus frequency of pesticides in control and experimental groups

In total 1000 cells were counted in every preparation. The ratio of PCE / MNPCE and the number of NCE were determined. Figure 1 shows micronucleus formation in bone marrow polychromatic erythrocytes (PCE, NCE and MNPCE). The Mann – Whitney U test was performed on the data obtained from the control and experimental groups, yielding significant results ($P < 0.05$). The results including the ratios of PCE / MNPCE and the number of NCE are presented in Figure 1 and Table 1. In the groups given α - endosulfan and β - endosulfan, a statistically significant increase was found in the numbers of PCE and MNPCE ($P < 0.05$). Also, a significant increase was observed in the number of micronuclei.

Table 1. Results of Micronucleus Test.

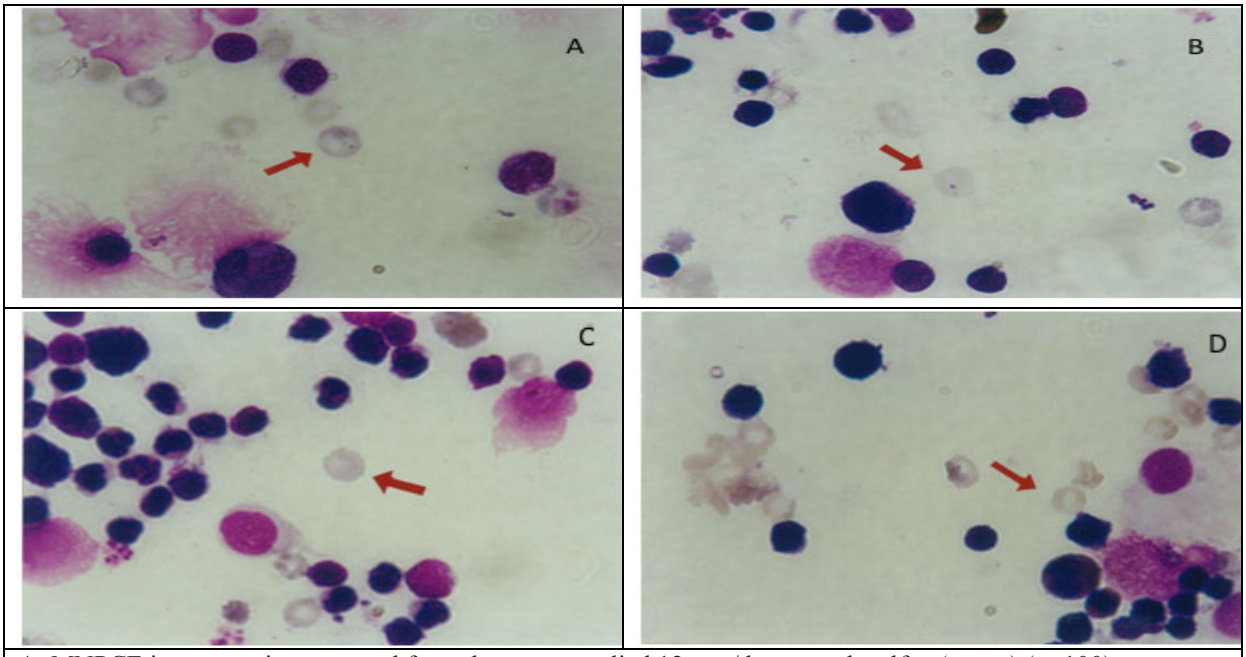
Experimental Group	Number	Erythrocytes					
		Total	Number of PCE	Percentage of PCE (%)	Number of NCE	Number of MNPCE	Percent age of MNPCE(%)
Control	5	17.000	11.371	67,0	5.541	88	0,52
α-Endosulfan (12 mg / kg)	2	6.000	4.193	69,8	1.701	106	1,77 *
α-Endosulfan (6 mg / kg)	4	12.000	8.160	68,0	3.698	142	1,18 *
β-Endosulfan (20 mg / kg)	4	12.000	8.341	69,5	3.498	161	1,34 *
β-Endosulfan (10 mg / kg)	4	11.000	7.425	67,5	3.463	112	1,01 *

*$P < 0.05$ was statistically significant. PCE: polychromatic erythrocytes;
NCE: normochromatic erythrocytes; MNPCE: micronucleated polychromatic erythrocytes.

The total number of PCE in rats of every group was divided by the group's total number of preparations, and an average PCE value for every group was calculated. Similarly, an average NCE value was determined, and then these calculations were compared to the data of the control group (Table 2).

Table 2. Average PCE and NCE Values of the Groups.

Groups	Average of PCE*	Average of NCE*
Control	669	331
α-Endosulfan (12 mg / kg)	699	301
α-Endosulfan (6 mg / kg)	680	320
β-Endosulfan (20 mg / kg)	695	305
β-Endosulfan (10 mg / kg)	675	325



A: MNPCE in preparation prepared from the group applied 12 mg / kg α - endosulfan (arrow) (× 100).
(MNPCE: micronucleated polychromatic erythrocytes)
B: MNPCE in preparation prepared from the group applied 20 mg / kg β - endosulfan (arrow) (× 100).
(MNPCE: micronucleated polychromatic erythrocytes)
C: PCE in preparation prepared from the group applied 6 mg / kg α - endosulfan (arrow) (× 100).
(PCE: polychromatic erythrocytes)
D: NCE in preparation prepared from the control group (arrow) (× 100).
(NCE: normochromatic erythrocytes)

Figure 1. Results Obtained from the Groups.

4. Discussion

Diseases and environmental problems caused by chemical products have been a serious concern. These chemicals enter our body through the food chain and exert various harmful effects. Endosulfan is among the chemicals widely used in agriculture that pose a risk to our health. Endosulfan, which can remain without degradation for a long time under natural conditions, leads to structural and functional abnormalities in the endocrine, reproductive, and immune systems (Han et al. 2015). It also has harmful effects on deoxyribonucleic acid (DNA) by damaging the genetic material in living beings (Taşlı et al. 2015).

Techniques used in the investigation of genotoxic effects of chemicals have significantly improved. The micronucleus test is one of the methods used for evaluating the clastogenic effects of chemical and physical agents (Şekeroğlu & Şekeroğlu 2011; Yüzbaşıoğlu et al. 2014). It is used in identifying genotoxicity and cancer screening (Üstüner 2011; Ambroise et al. 2013). A study investigating the genotoxic effects of preservatives used in daily life with the micronucleus test reported that preservatives used in food frequently act as carcinogens by exerting a positive genotoxic effect (Yüzbaşıoğlu et al. 2014). Many studies on cancer and genomic irregularity in living beings exposed to physical and chemical agents in aquatic and terrestrial environments reported a significantly high MNPCE frequency (Sağır 2012; Dönmez & Yılmaz 2015; Özdemir et. al. 2015; Taşlı et. al. 2015). Therefore, the micronucleus test has become a common test used in genetic toxicology researches for implementation in vitro and in vivo on all cell types that have completed one mitosis.

An increase in the number of PCE cells in the bone marrow and the percentage of MNPCE due to pesticide application is an indication of toxicity (Soheir & Ezzat 1985; Demirel & Zaman 2002). It has been demonstrated that endosulfan applied to rats intraperitoneally leads to an increase in the number of PCE and MNPCE (Soheir & Ezzat 1985; Wolfe et. al. 1980; Syhanco & Usha 1983; Kolonkaya 1988). An increase in the number of MNPCE is an indication of a clastogenic effect, (Misawa et. al. 1982; Kolonkaya 1987) which has a negative influence on both individuals and their future generations.

This study found that both isomers of endosulfan increased the numbers of PCE and MNPCE in rats and hence exerted a clastogenic effect ($P < 0.05$), which was consistent with the other researchers' findings. The rate of formation of PCE is higher for the isomer exerting more clastogenic effect. Based on the results obtained, the order for the formation of PCE was as follows: α - endosulfan 12 mg / kg, β - endosulfan 20 mg / kg, α - endosulfan 6 mg / kg, and β - endosulfan 10 mg / kg ($P < 0.05$). The orders for percentage of MNPCE and the ratios of total erythrocytes to PCE, the ratio of total erythrocytes to MNPCE, or the ratio of total erythrocytes to NCE, were also similar ($P < 0.05$).

Same was the order for clastogenic effect. α - Endosulfan 12 mg / kg had the highest average PCE and caused the maximum toxicity, followed by β - endosulfan 20 mg / kg, α - endosulfan 6 mg / kg, and β - endosulfan 10 mg / kg. The average NCE is the average of normal erythrocytes with no toxicity. The average NCE of the treatment group was different from the average NCE of the control group. This difference is because of the lethal effect of endosulfan.

5. Conclusion

The present study showed that α and β isomers of endosulfan exert a clastogenic effect in the bone marrow leading to toxicity in rats. Commonly used pesticides are very dangerous for living beings. If they continue to be used uncontrollably, they may cause environmental pollution in the future. Moreover, they may lead to very serious health problems, in particular clastogenic effect through bioaccumulation in the living beings. The assessment of the clastogenic effects of pesticides using the micronucleus test will increase people's awareness about the harmful consequences if these chemicals are not carefully used.

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