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Determination of the genotoxic effects of various dioxins by *Drosophila* wing spot test

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Dioxins, furans and other polychlorinated biphenyls (coplanar PCBs) are three structural and toxicologically related families of compounds classified as the most toxic synthetic chemical. In this study, we investigated possible genotoxic effects of polychlorinated dibenzo-p-dioxins (PCDDs), man-made environmental contaminant, in *Drosophila melanogaster* by somatic mutation and recombination test (SMART). In this, we observe the chemical effects on wing phenotype of the transheterozygote flies carrying marker gene. Lethal doses of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8,9-H_XCDD, and 1,2,3,4,6,7,8,9-OCDD were determined. Doses of 1×10^{-7}, 2.5×10^{-7}, 5×10^{-7}, and 10×10^{-7} µg/mL of PCDDs were used. In addition, the observed mutations were classified according to the size and the type of the mutations per wing. Results revealed no significant genotoxic effect of any of the dioxins tested. According to the mechanisms involved in the antigenotoxicity of PCDDs, it is suggested that the observed effects can be linked to the differences in the aryl hydrocarbon receptor (AHR) amino acid sequences in the gene protein of *D. melanogaster*.

Keywords: Aryl hydrocarbon receptor, Polychlorinated dibenzo-*p*-dioxins, Somatic mutation and recombination test

Polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) constitute a group of persistent environmental chemicals[1]. These compounds are formed as unwarranted byproducts in a variety of chemical and thermal processes, and except for scientific research, they are of no economic importance[2]. They cause various toxicological and biological responses typified by dermal toxicity, reproductive effects, teratogenicity, thymic atrophy, endocrine disruption and carcinogenicity and induction of xenobiotic metabolizing enzymes in experimental animals[3,4]. These dioxin-like compounds (DLCs) and dioxins with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) being the prototype are one of the best characterized chemicals causing various kinds of toxicity[5]. Yoshioka & Tohyama have discussed the mechanism of the TCDD toxic effect in their review[6]. TCDD brings about a wide variety of toxic and biochemical effects via aryl hydrocarbon receptor (AhR)-mediated signaling pathways[6,7]. Acute exposure to high dose TCDD results in oxidative stress in multiple tissues and species[8].

Drosophila melanogaster has great importance in genetics and cell biology and also in toxicological studies[9]. In the present work, we tried to evaluate the genotoxic potential of the PCDDs (2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8,9-H_XCDD, and 1,2,3,4,6,7,8,9-OCDD) against transheterozygote *Drosophila* larvae using wing somatic mutation and recombination test (SMART).

Material and Methods

Chemicals

Dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, CAS no. 1746-01-6), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD, CAS no. 40321-76-4), 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin (H_XCDD, CAS no. 19408-74-3), and 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD, CAS no. 3268-87-9) were obtained from AccuStandard (New Haven, USA). Ethyl methanesulfonate (EMS, CAS no. 62-50-0) and dimethyl sulphoxide (DMSO, CAS no. 67-68-5) were obtained from Sigma Chemical (St. Louis, MO). Prior to use, all the dioxins were dissolved in 1% dimethyl sulphoxide.

Strains

In our study, *mwh* (*mwh/mwh*) and flr^3 (flr^3/*In* (3LR) *TM3* Bd^S) mutant strains of *Drosophila* have been used. These mutant strains carry determinant genes. Of these determinant genes, the *flare* (flr^3, 3-38.8) gene forms dulled, points like hair instead of the normal long and straight feathers on the wings. Since the *flare* gene in its homozygote state causes lethal effects in the embryonic stage, it is used together with the stabilizing *TM3* chromosome in order to protect the individuals from the embryonic lethal effects of the *flare* gene and to suppress the recombination. The other determinant gene *mwh* (*mwh*, 3-0.3) shows itself by causing the wing hair

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to come out as three or more from the same cell[10]. This stock had been maintained for many years in the Laboratory at the Department of Biology of the Atatürk University in Erzurum, Turkey. Therefore, it is highly inbred with little genetic variation.

Experimental procedures

At first, *mwh* virgin females and *flr*^{3} males of mutant strains were crossbred eggs were collected in periods of 8 h. The transheterozygote larvae obtained from these eggs after 72±4 h were placed in application tubes containing four different concentrations of dioxin (1×10^{-7}; 2.5×10^{-7}; 5×10^{-7}, and 10×10^{-7} µg/mL) and *Drosophila* instant medium. The larvae were kept in this medium until they matured. The mature individuals were collected and kept in 70% alcohol at 4°C until their wing prepares were readied. The wing prepares prepared by separating according to normal and serrate wing phenotype. The wings (both the dorsal and ventral surface) were examined under the light microscope (400X) by separating into segments and the mutant clones detected were recorded[10]. These clones were classified as small single type (1-2 cells), large single type (>2) and twin clones[11]. Aside from the experimental groups including dioxin, positive control (1.0 mM EMS) and negative control (distilled water and DMSO) groups were also prepared. All experiments were kept at a temperature of 25°C and 40-60% relative humidity.

Statistical analysis

The data were evaluated according to the multiple decision procedures proposed by Frei and Würgler resulting in four possible diagnoses: positive, negative, inconclusive or weakly positive[11,12]. The relative frequencies of each group of spots were compared with the respective negative control using the conditional binomial test of Kastenbaum & Bowman[13].

Results and Discussion

The SMART was used to observe the effects of PCDDs on the wing phenotype of trans-heterozygote flies carrying a marker gene. The analysis of the wing spot data from chronic treatments shows the lack of twin spots, which are produced by mitotic recombination exclusively. A total of 80 wings each with the normal wing (*mwh/flr*^{3}) and serrate wing (*mwh/TM3*) phenotype were examined for each application group.

The results from the experiments of the genotoxicity of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8,9-H_XCDD, and 1,2,3,4,6,7,8,9-OCDD in the SMART assay are shown in Tables 1 and 2, including data from both marker-heterozygous (*mwh/flr*^{3}) and balancer-heterozygous (*mwh/TM3*) flies. Tables 1 & 2

Table 1 — Genotoxicity of the polychlorinated dibenzo-*p*-dioxins (PCDD) in the *D. melanogaster* wing spot test results obtained with *mwh/flr*^{3} wings

Experimental groups	N	Small single spots (1–2 cells) (*m = 2*)			Large single spots (>2 cells) (*m = 5*)			Twin spots (*m = 5*)			Total *mwh* spots (*m = 2*)			Total spots (m = 2)			(CIF)
		No	Fr.	*D*	No	Fr.	*D*	No	Fr.	*D*	No	Fr.	*D*	No	Fr.	*D*	
Distilled water	80	14	(0.18)		4	(0.05)		2	(0.03)		17	(0.21)		20	(0.25)		0.87
DMSO (1%)	80	17	(0.21)	i	5	(0.06)	-	2	(0.03)	i	20	(0.25)	i	24	(0.30)	i	1.02
EMS (1mM)	80	42	(0.53)	+	28	(0.35)	+	14	(0.18)	+	70	(0.88)	+	84	(1.05)	+	3.58
2,3,7,8-TCDD 1×10^{-7}µg/mL	80	11	(0.14)	-	3	(0.04)	-	1	(0.01)	i	13	(0.16)	-	15	(0.19)	-	0.66
2,3,7,8-TCDD 2.5×10^{-7}µg/mL	80	19	(0.24)	i	4	(0.05)	-	2	(0.03)	i	19	(0.24)	-	25	(0.31)	-	0.97
2,3,7,8-TCDD 5×10^{-7}µg/mL	80	21	(0.26)	i	7	(0.09)	-	4	(0.05)	i	24	(0.30)	i	32	(0.40)	i	1.22
2,3,7,8-TCDD 10×10^{-7}µg/mL	80	23	(0.29)	i	9	(0.11)	i	5	(0.06)	i	31	(0.39)	i	37	(0.46)	i	1.58
1,2,3,7,8-PeCDD 1×10^{-7}µg/mL	80	11	(0.14)	-	3	(0.04)	-	1	(0.01)	i	13	(0.16)	-	15	(0.19)	-	0.66
1,2,3,7,8-PeCDD 2.5×10^{-7}µg/mL	80	10	(0.13)	-	4	(0.05)	-	2	(0.03)	i	14	(0.18)	-	16	(0.20)	-	0.71
1,2,3,7,8-PeCDD 5×10^{-7}µg/mL	80	19	(0.24)	i	4	(0.05)	-	2	(0.03)	i	19	(0.24)	-	25	(0.31)	-	0.97
1,2,3,7,8-PeCDD 10×10^{-7}µg/mL	80	21	(0.26)	i	7	(0.09)	-	4	(0.05)	i	24	(0.30)	i	32	(0.40)	i	1.22
1,2,3,7,8,9-HxCDD 1×10^{-7}µg/mL	80	8	(0.10)	-	2	(0.03)	-	0	(0.00)	-	9	(0.11)	-	10	(0.13)	-	0.46
1,2,3,7,8,9-HxCDD 2.5×10^{-7}µg/mL	80	9	(0.11)	-	2	(0.03)	-	1	(0.01)	i	11	(0.14)	-	11	(0.14)	-	0.56
1,2,3,7,8,9-HxCDD 5×10^{-7}µg/mL	80	15	(0.19)	-	4	(0.05)	-	1	(0.01)	i	11	(0.14)	-	19	(0.24)	-	0.56
1,2,3,7,8,9-HxCDD 10×10^{-7}µg/mL	80	17	(0.21)	-	5	(0.06)	-	2	(0.03)	i	14	(0.18)	-	22	(0.28)	-	0.71
1,2,3,4,6,7,8,9-OCDD 1×10^{-7}µg/mL	80	6	(0.08)	-	2	(0.03)	-	0	(0.00)	-	8	(0.10)	-	8	(0.10)	-	0.40
1,2,3,4,6,7,8,9-OCDD 2.5×10^{-7}µg/mL	80	7	(0.09)	-	2	(0.03)	-	0	(0.00)	-	8	(0.10)	-	9	(0.11)	-	0.40
1,2,3,4,6,7,8,9-OCDD 5×10^{-7}µg/mL	80	8	(0.10)	-	3	(0.04)	-	1	(0.01)	i	10	(0.13)	-	12	(0.15)	-	0.51
1,2,3,4,6,7,8,9-OCDD 10×10^{-7}µg/mL	80	10	(0.13)	-	3	(0.04)	-	1	(0.01)	i	13	(0.16)	-	14	(0.18)	-	0.66

[N, Number of wings; No, number of clones; Fr., frequency; D, statistical diagnosis according to Frei and Würgler [11]; CIF, Frequency of clone formation per 10^5 cells; +, positive; -, negative; i, inconclusive; m, multiplication factor; probability levels $\alpha=\beta=0.05$]

Table 2 — Genotoxicity of the polychlorinated dibenzo-*p*-dioxins (PCDD) in the *D. melanogaster* wing spot test results obtained with *mwh/TM3* wings

Experimental groups		N	Small single spots (1–2 cells) (*m = 2*)			Large single spots (>2 cells) (*m = 5*)			Twin spots (*m = 5*)			Total *mwh* spots (*m = 2*)			Total spots (m = 2)			(CIF)
			No	Fr.	*D*	No	Fr.	*D*	No	Fr.	*D*	No	Fr.	*D*	No	Fr.	*D*	
Distilled water		80	12	(0.15)		3	(0.04)					15	(0.19)		15	(0.19)		0.76
DMSO (1%)		80	15	(0.19)	i	3	(0.04)	i		*		18	(0.23)	i	18	(0.23)	i	0.92
EMS (1.0 mM)		80	40	(0.50)	+	25	(0.32)	+				65	(0.82)	+	65	(0.82)	+	3.32
2,3,7,8-TCDD	1×10^{-7} µg/mL	80	11	(0.14)	-	3	(0.04)	i				14	(0.18)	-	14	(0.18)	-	0.71
	2.5×10^{-7} µg/mL	80	18	(0.23)	i	3	(0.04)	i				21	(0.26)	i	21	(0.26)	i	1.07
	5×10^{-7} µg/mL	80	20	(0.25)	i	6	(0.08)	i				26	(0.32)	i	26	(0.32)	i	1.33
	10×10^{-7} µg/mL	80	21	(0.26)	i	7	(0.09)	i				28	(0.35)	i	28	(0.35)	i	1.43
1,2,3,7,8-PeCDD	1×10^{-7} µg/mL	80	10	(0.13)	-	2	(0.03)	-				12	(0.15)	-	12	(0.15)	-	0.61
	2.5×10^{-7} µg/mL	80	10	(0.13)	-	2	(0.03)	-				12	(0.25)	-	12	(0.25)	-	0.61
	5×10^{-7} µg/mL	80	16	(0.20)	i	3	(0.04)	i				19	(0.24)	-	19	(0.24)	-	0.97
	10×10^{-7} µg/mL	80	18	(0.23)	i	4	(0.05)	i				22	(0.28)	i	22	(0.28)	i	1.12
1,2,3,7,8,9-HxCDD	1×10^{-7} µg/mL	80	8	(0.10)	-	0	(0.00)	-				7	(0.09)	-	7	(0.09)	-	0.35
	2.5×10^{-7} µg/mL	80	9	(0.11)	-	0	(0.00)	-				7	(0.09)	-	7	(0.09)	-	0.35
	5×10^{-7} µg/mL	80	10	(0.13)	-	2	(0.03)	-				10	(0.13)	-	10	(0.13)	-	0.51
	10×10^{-7} µg/mL	80	13	(0.16)	-	2	(0.03)	-				11	(0.14)	-	11	(0.14)	-	0.56
1,2,3,4,6,7,8,9-OCDD	1×10^{-7} µg/mL	80	7	(0.09)	-	0	(0.00)	-				7	(0.09)	-	7	(0.09)	-	0.35
	2.5×10^{-7} µg/mL	80	8	(0.10)	-	0	(0.00)	-				8	(0.10)	-	8	(0.10)	-	0.40
	5×10^{-7} µg/mL	80	9	(0.11)	-	1	(0.01)	-				10	(0.13)	-	10	(0.13)	-	0.51
	10×10^{-7} µg/mL	80	11	(0.14)	-	2	(0.03)	-				13	(0.16)	-	13	(0.16)	-	0.66

[N, Number of wings; No, number of clones; Fr., frequency; D, statistical diagnosis according to Frei & Würgler[11]; CIF, Frequency of clone formation per 10^5 cells; *, balancer chromosome *TM3* does not carry the *flr*^{3} mutation.+, positive; -, negative; i, inconclusive; m, multiplication factor; probability levels α=β=0.05]

show that no positive result was observed for the individuals of the polychlorinated dibenzo-*p*-dioxins groups with normal and serrate wings except for EMS. When all clone frequencies are examined, it is observed that the results are similar to the dimethyl sulphoxide control group.

While the use of *D. melanogaster* for evaluation of genotoxicity is well established as a test system, the somatic mutation and recombination test (SMART) was used for the first time in the study for determination of the genotoxic effects of dioxins. Due to a genome similarity compared to mammals and easy maintenance in the laboratory, these flies represent an appropriate organism to run *in vivo* short-term tests[14,15]. SMART is a simple and fast short-term assay compared with other *in vivo* tests. It is effortless to conduct and effective in the detection of a wide range of aspects of genetic alterations[10,16]. Through the use of these test systems, it is possible to evaluate the genotoxic activity of a single compound as well as complex mixtures[14]. Analysis of the MH descendants (marked trans heterozygous descendants, *mwh/flr*^{3}) and of the BH descendants (balanced heterozygote descendants, *mwh/TM3*) has demonstrated that it is possible to quantify the recombinogenic events in the total of mutant spots detected[17,18]. Thus, due to its capabilities, SMART was chosen to evaluate the genotoxic effects of the dioxins.

In classic genotoxicity studies investigating the effect of dioxins on various organisms, the literature is available to negative results as well as positive results[19-21]. There is a review focusing on the latest progress reported on developmental toxicity mechanisms in terms of teratogenicity, malformation and morphological changes in laboratory animals exposed to TCDD[5,6,22]. In a study, two mixtures of polychlorinated biphenyls (PCB) were fed to adults or larvae of *D. melanogaster.* Genetic tests were performed on the loss of sex chromosomes as a measure of a chromosome breaking action and on nondisjunction of the sex chromosomes. The results did not indicate any effect by the PCB mixtures[23]. Again, adults of *D. melanogaster* were exposed to different concentrations TCDD (50, 250, and 500 ppm) in the sex-linked recessive lethal test, and any mutation in the germ cells of male individuals was not observed[24].

In another study, the genotoxic effects in two patients with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) intoxication were measured. Sister chromatid exchange (SCE), micronuclei (MN) and comet assay tail factor in peripheral blood lymphocyte of the patients and of control persons were determined. Within a period of 13 months, MN had returned to a nearly normal range in both patients. SCE had been within normal ranges all the time. It was also determined the comet assay tail factor (DNA damage level) in peripheral blood lymphocyte of the patients. According to Valic *et al.*,[25], this delayed and transient effect seems to indicate some kind of ‘‘indirect’’ or ‘‘secondary’’ genotoxic effect of TCDD. However, it was concluded that DNA damaging effects, caused directly or indirectly, could be an indicator for a possible carcinogenic risk of TCDD. In another study, in order to evaluate the genotoxicity of three chemicals (2,3,7,8-TCDD, 2,3,4,7,8-PenCDF, and 3,4,5,3',4'-Co-PenCB) were examined their effects on the induction of sister chromatid exchanges (SCEs), which were frequently utilized as an indicator of biological and genetic damage due to exposure to carcinogens or mutagens, in cultured human lymphocytes in the absence or presence of 7,8-benzoflavone (ANF). TCDD, PenCDF, and Co-PenCB significantly increased the frequency of SCEs (as an indicator of the genotoxic potency) with almost the same dose-dependent manner in terms of the concentration of TCDD toxic equivalent[26].

Our laboratory has previously showed that PCDDs are powerful inducers of longevity and some developmental parameters in *D. melanogaster*. It has been reported in laboratory animals that oxidative stress caused by TCDD exposure leads to an increase in reactive oxygen species (ROS), lipid peroxidation and DNA damage production[27,28]. In addition, there have been several investigations showing that dioxins play a specific role in cancer initiation and promotion, not have direct genotoxic activity. Dioxins and dioxin-like chemicals demonstrate high-affinity binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, which mediates most, if not all, of the toxic responses of these agents[29,30]. In a review, the latest techniques for the detection and real time monitoring of dioxins, furans and related compounds in gaseous or liquid phases were based on optical and spectroscopic methods looking at future perspectives[22].

Ah receptor, suggesting that an important factor in developmental and homeostatic processes are much evidence. Ah receptor is a member of the bHLH-PAS (basic helix-loop-helix/Per-Arnt-Sim) family of transcriptional regulatory proteins[31]. However, as an exception, this receptor does not bind dioxin in *Caenorhabditis elegans* (nematode), *Mya arenaria* (mollusks) and *D. melanogaster* (fruit fly) due to the difference in the amino acid sequences of the gene of the receptor protein, which is the homolog of the Ah receptor in the vertebrates[32]. The homologue of the Ah receptor and the aryl nuclear translocase (Arnt) in the vertebrates are the Spineless (Ss) and Tango (Tgo) proteins in *D. melanogaster*, respectively. The Ah receptor has two domains, PAS-A and PAS-B. *Drosophila*, like other invertebrates, does not suffer dioxin toxicity because its Ahr homologue (Ss) does not bind dioxins due to the amino acid sequence in the PAS-B domain of invertebrates is different from that of the vertebrates[33-37]. In previously our study, survival rates and longevity of same application groups were compared to the control group for evaluation of detected toxic effects. In all application groups, both the survival rate and each population’s longevity decreased, depending on the concentration of dioxins[38]. However, in this study, PCDDs have not been shown to be genotoxic at larval stages of *D. melanogaster*. The literature also supports the fact that no mutagenic effect was observed in this study in which the genotoxic effect of dioxins was investigated by wing spot test.

Conclusion

As a summary, the results of our study show in *Drosophila* that PCDDs (2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8,9-H_XCDD, and 1,2,3,4,6,7,8,9-OCDD) are not able to produce genotoxic effects, as least as measured in the wing spot test (SMART). Although 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8, 9-H_XCDD, and 1,2,3,4,6,7,8,9-OCDD are not statistically significant genotoxic on *Drosophila*, in our previous studies, the possible observed cytotoxic effect of the dioxins may be attributed to oxidative stress induced by free radical production that results in indirect DNA damage.

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Conflict of interest

Authors declare no competing interests.

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