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Examination of the Genotoxic Effects of Various Parabens Used as Food Additives with the *Drosophila* Wing Spot Test (SMART)

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Abstract

The objective of this study is to determine the possible genotoxic effects of the para-hydroxybenzoic acid esters (Parabens) of ethylparaben and butylparaben used as preservative substances in the food, cosmetic and drug industries on *Drosophila melanogaster* with the Somatic Mutation and Recombination Test (SMART). In our study, two different mutant strains of *D. melanogaster* known as the fruit fly with recessive *flr*^{3} and *mwh* identifier genes in its genome were used. The trans-heterozygous larvae of 72±4 hours obtained as a result of the crossbreeding between these two mutant strains were fed chronically with various concentrations (100, 150, 200 and 250mM) of ethylparaben and butylparaben. In addition, experimental setups for control groups have been prepared using ethyl methanesulfonate (EMS) and distilled water. Wing preparates of the full-grown individuals developed from these larvae have been prepared and these preparates have been examined under a microscope. According to the data obtained from microscopic analysis, no significant increase has been determined in all type clone number of ethylparaben and butylparaben groups in comparison with the control group. This has been determined to have negative (-) or insignificant (i) effects on the control group statistically ($P>0.05$). As a result of the data we obtained, it has been concluded that parabens show toxic effects, although no genotoxic effects on *D. melanogaster*.

Keywords: *Drosophila melanogaster*, ethylparaben, butylparaben, genotoxicity, SMART

INTRODUCTION

With the advent of technology, issues such as the development of different production methods in the food sector, the increase in the variety of food products, the desire to consume seasonal foods in every season and to increase the shelf life have made it obligatory to use Food Additives (FA) [1]. Parallel to the industrialization that commenced in the 19th century, an increase has been observed in FA use and has today reached up to 200.000 tons annually. According to a study that has been carried out, people are subject to 5-6 kg of additive substances per year in western countries where processed food consumption has a ratio of approximately 75% [2].

Protective FA are defined as chemical substances that protect food products from deterioration caused by various microorganisms therefore increasing their shelf lives [3]. For this aim, many food additives are used frequently in the food industry. One of these food additive groups is the parabens (para-hydroxy benzoic acids).

Parabens are a group of chemicals that are widely used as preserving additive substances in the food, cosmetic and drug industries. The most widely used parabens are methylparaben, ethylparaben, propylparaben and butylparaben. They have been defined as ideal preserving substances due to the fact that they have a wide antimicrobial effect spectrum, they are safe to use, can stay within a wide pH interval, cause less irritation in comparison with other substances and are less toxic [4]. Parabens are frequently used in bakery products (cakes, bread crust, fillers etc.), drinks, fish, aroma extracts, fruit products, gelatine, jam, gel, malt extracts, olives, pickles, salad sauces, syrups and wine.

The discussion regarding the safety of parabens has been going on for years within the scientific community. The toxic effects of prabens have been tried to be determined in many past *in vivo* and *in vitro* acute and chronic toxicity [5, 6], mutagenity [7], teratogenity [8] and cytotoxicity [9] studies. Many of these studies have put forth results stating that parabens are not toxic and that they can be used safely. However, there are studies stating that parabens might be toxic and mutagenic due to their estrogenic activities [10, 11]. In addition, with the determination of parabens scraps in some breast tumours [12] and various news in the media regarding information that parabens are hazardous to human health, problems regarding the reliability of these substances have resurfaced.

Today, it is now known whether many FA including parabens have toxic effects or not and these substances continue to be used recklessly. Therefore, scientists are trying to determine the clastogenic, mutagenic and genotoxic effects of FAs by carrying out various *in vivo* and *in vitro* test methods. *In vivo* mutagenity tests are generally carried out on insects and mammals. One of the most widely used genotoxicity tests carried out on insects is the wing somatic mutation and recombination test carried out with *Drosophila*. This test method can create a link between microorganism *in vitro* and mammal *in vivo* genotoxicity test systems [13]. SMART enables the determination of the genetic results of various chromosome aberrations such as point mutation, deletion, translocation, somatic recombination and chromosome loss or non-disjunction [14].

The objective of this study is to determine the possible genotoxic effects of the parabens ethylparaben and

butylparaben used as preservatives in the food, cosmetic and drug industry on *D. melanogaster* through wing Somatic Mutation and Recombination Test (SMART).

MATERIALS AND METHODS

Chemicals

The ethylparaben (99.0% purity, CAS No. 120-47-8), butylparaben (99.0% purity, CAS No. 94-26-8), ethyl methanesulfonate (100% purity, CAS no. 62-50-0) and ethyl alcohol (99.5% purity, CAS No. 64-17-5) were obtained from the Sigma-Aldrich Company (St Louis, Missouri, USA), while Drosophila instant medium was obtained from the Carolina Biological Supply Company (2700 York Road, Burlington, USA).

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, point 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 [14]. The other determinant gene *mwh* (*mwh*, 3-0.3) shows itself by causing the wing hair to come out as three or more from the same cell. For genetic symbols and description, see Lindsley and Zimm [15].

Treatment procedure

LD_{50} concentrations of parabens have been determined by carrying out pre-studies. These concentrations are 300mM for ethylparaben and butylparaben. Whereas the application doses have been selected to be lower than the determined LD_{50} concentrations. Afterwards, *flr*^{3} virgin females and *mwh* males of mutant strains were crossbred eggs were collected in periods of 8 hours. The trans-heterozygous larvae obtained from these eggs after 72±4 hours were placed in application tubes containing 4 different concentrations (100, 150, 200 and 250mM) of paraben solution and *Drosophila* instant medium. The larvae were kept inside this feed lot until they matured. The mature specimens were collected and kept in 70% alcohol at +4°C until their wing slides were readied. The wing slides prepared by separating according to normal and serrate wing phenotype were examined under the light microscope (400X) by separating into segments and the mutant clones detected were recorded. These clones were classified as small single type (1-2 cells), large single type (>2) and twin clones. Aside from the experimental groups including paraben, positive control (1mM EMS) and negative control (distilled water) groups were also prepared.

Statistical analysis

For statistical calculations, the conditional binomial test according to Kastenbaum and Bowman was used with 5% significance levels [16]. Statistical comparisons of survival rates were made by using Chi-square test for ratios for independent samples. The differences between groups were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

When the ethylparaben and butylparaben application groups are compared with the control group, no genotoxic effect is observed in our study (Table 1 and 2). As can be seen in Table 1 and 2, no positive result was observed for the individuals of the paraben groups with normal and serrate wings except for EMS. When all clone frequencies are examined, it is observed that the results are similar with the distilled water control group. When Table 1 and 2 are examined, no increase has been observed in the small single type clone numbers for all application groups. Even though large single type clone number has increased with concentration especially in high concentrations groups of the ethylparaben and butylparaben (200 and 250mM), this ratio has been determined to be statistically insignificant ($P>0.05$).

Table 1. Wing spot test data obtained with the parabens tested. Results with *mwh/flr*^{3} wings

Application groups (mM)	Number of wings (N)	Small single spots (1–2 cell) *(m = 2)*			Large single spots (>2 cell) *(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*	
Control	80	12	(0.15)		1	(0.01)		1	(0.01)		14	(0.18)		14	(0.19)		0.71
1 EMS	80	49	(0.61)	+	30	(0.38)	+	19	(0.24)	+	84	(1.05)	+	98	(1.23)	+	4.30
Ethylparaben																	
100	80	4	(0.05)	-	1	(0.01)	i	0	(0.00)	i	5	(0.06)	-	5	(0.06)	-	0.25
150	80	4	(0.05)	-	1	(0.01)	i	1	(0.01)	i	5	(0.06)	-	6	(0.08)	-	0.25
200	80	8	(0.10)	-	2	(0.03)	i	1	(0.01)	i	10	(0.13)	-	11	(0.14)	-	0.51
250	80	11	(0.14)	i	2	(0.03)	i	0	(0.00)	i	13	(0.16)	i	13	(0.16)	i	0.66
Butylparaben																	
100	80	9	(0.11)	-	1	(0.01)	i	0	(0.00)	i	10	(0.13)	-	10	(0.13)	-	0.51
150	80	8	(0.10)	-	1	(0.01)	i	1	(0.01)	i	10	(0.13)	-	10	(0.13)	-	0.51
200	80	12	(0.15)	i	2	(0.03)	i	1	(0.01)	i	14	(0.20)	i	15	(0.19)	i	0.71
250	80	11	(0.14)	i	3	(0.04)	i	0	(0.00)	i	14	(0.21)	i	14	(0.18)	i	0.71

No: Number of clones; Fr: frequency; D: statistical analysis according to Frei and Würgler [13]; +: positive; –: negative; i: inconclusive; m: multiplication factor; probability levels $\alpha = \beta = 0.05$, CIF: Frequency of clone formation per 10^5 cell.

Table 2. Wing spot test data obtained with the parabens tested. Results with *mwh/TM3* wings

Application groups (mM)	Number of wings (N)	Small single spots (1–2 cell) *(m = 2)*			Large single spots (>2 cell) *(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	
Control	80	9	(0.11)		1	(0.01)					10	(0.13)		10	(0.13)		0.51
1 EMS	80	44	(0.56)	+	21	(0.27)	+				65	(0.81)	+	65	(0.81)	+	3.33
Ethylparaben																	
100	80	6	(0.08)	-	0	(0.00)	i				6	(0.08)	-	6	(0.08)	-	0.30
150	80	8	(0.10)	i	0	(0.00)	i				8	(0.10)	-	8	(0.10)	-	0.40
200	80	10	(0.13)	i	1	(0.01)	i	Balancer chromosome *TM3* does not carry the *flr*^{3} mutation			11	(0.14)	i	11	(0.14)	i	0.56
250	80	11	(0.14)	i	1	(0.01)	i				12	(0.15)	i	12	(0.15)	i	0.61
Butylparaben																	
100	80	9	(0.11)	i	0	(0.00)	i				9	(0.11)	i	9	(0.11)	i	0.46
150	80	10	(0.13)	i	0	(0.00)	i				10	(0.13)	i	10	(0.13)	i	0.51
200	80	10	(0.13)	i	1	(0.01)	i				11	(0.14)	i	11	(0.14)	i	0.56
250	80	12	(0.15)	i	1	(0.01)	i				13	(0.16)	i	13	(0.16)	i	0.66

No: Number of clones; Fr: frequency; D: statistical analysis according to Frei and Würgler [13]; +: positive; –: negative; i: inconclusive; m: multiplication factor; probability levels $\alpha = \beta = 0.05$, CIF: Frequency of clone formation per 10^5 cell.

While in line with the increase in concentration the Clone Induction Frequencies (CIF) for the normal wing phenotype of ethylparaben application groups are 0.25, 0.25, 0.51 and 0.66 (Table 1) respectively, these ratios for the serrate phenotype are 0.30, 0.40, 0.56 and 0.61 respectively (Table 2). The CIF values for the normal wing phenotype in the butylparaben application group are 0.51, 0.51, 0.71 and 0.71 (Table 1) respectively, whereas for the serrate wing phenotype the ratios are 0.46, 0.51, 0.56 and 0.66 (Table 2) respectively. CIF values for the distilled water negative control group were determined as 0.71 for normal wing and 0.51 for serrate wing.

The results of percentages of survival reported for parabens are shown in Table 3. The survival rates of treatment groups were compared with the control group (98%) for evaluation of detected toxic effects. In the study we carried out, in the application groups belonging to all concentrations (100, 150, 200 and 250mM) it was observed that the used parabens became toxic *D. melanogaster* larvae. The results show that the lowest survival rate was in the 250mM butylparaben application group (74%) (Table 3).

Table 3. Survival rate of the flies exposed to different concentration of parabens

Compounds	Concentration (mM)	Survival (%)
Control	Distilled water	98
Ethylparaben	100	98
	150	92
	200	85*
	250	80*
Butylparaben	100	95
	150	91
	200	87*
	250	74*

* $P < 0.05$, survival statistics (Chi-square test)

It is still being discussed in the scientific world whether the many additives used in the food industry have toxic effects or not. When the amount of substances applied to the foods and the number of people subject to them are considered, the importance of this issue is clearly understood. Therefore, scientists have been trying for years to determine the possible clastogenic, mutagenic and genotoxic effects of many food additives via *in vivo* and *in vitro* test methods.

According to the results of a study in which the genotoxic effects of 39 different FA have been examined, it has been determined that 7 food paints (amaranth, allura red, new coccine, tartrazine, erythrosine, phloxine and rose bengal), 2 antioxidant FAs (butylated hydroxyanisole and butylated hydroxytoluene), 3 fungicides (biphenyl, sodium o-phenylphenol and thiabendazole) and 4 food sweeteners (sodium cyclamate, saccharin, sodium saccharin and sucralose) have caused DNA damage in the digestive organs of mice causing genotoxic effects [17].

It has been determined that preservative FA such as sodium nitrate, potassium nitrite and potassium nitrate decrease the average life span of *D. melanogaster* at 75mM concentrations [18]. In another study examining the genotoxic effects of the same substances via SMART, it has been determined that all application groups display genotoxic effects at 50, 75 and 100mM concentrations whereas the groups obtained from a mixture of these substances display genotoxic effects at 25mM concentration [19].

It has been determined that benzoic acid used as a preserving additive in foods increases wing spot mutations in *D. melanogaster* in comparison to the control group [20]. In addition, it has also been determined that benzoic acid increases chromosome aberrations in *Allium sativum* root cells thereby significantly decreasing the mitotic index [21]. However, there are also many studies in the literature stating that benzoic acid is not genotoxic [22, 23]. Türkoğlu [24] has stated that the food preservatives sodium benzoate (SB), boric acid (BA), citric acid (CA), potassium citrate

(PC) and sodium citrate (SC) showed genotoxic effects on root tips of *Allium cepa*.

In the study carried out by Schlatter et al. [25] in which they examined the possible genotoxic effects via SMART of food preserving substances potassium sorbate, sodium sorbate and 4, 5-epoxy-2-hexenoic acid, they determined that only 4,5-epoxy-2-hexenoic acid has a weak genotoxic effect and that potassium sorbate and sodium sorbate displayed no genotoxic effects.

After the determination of parabens in human breast cancer tissue, its relationship with cancer has been the subject of intensive studies. Recent studies have shown the effectiveness of the increase in the incidence of breast cancer, the preventive effects of human reproduction functions and the oestrogenic stimulus in malignant melanoma [26, 27]. However, there are also studies showing that paraben has been detected in the urines of male and female individuals who have not been subject to parabens and that this substance has accumulated due to previous contacts [28]. All these results have brought up some anxieties regarding the safe use of parabens as antimicrobial preservatives.

According to the risk evaluation prepared by "The European Food Safety Authority" (EFSA) in 2004, new studies carried out using methylparaben have put forth that a body weight dose of 300mg/kg daily for rabbits and 550mg/kg daily for rodents does not cause any toxicity on the foetus [29]. In the 2005 risk evaluation of the "Ec Scientific Committee on Consumer Products" (SCCP), according to acute, subacute and chronic toxicity studies, parabens have not been evaluated as toxic, carcinogenic and teratogenic and the Acceptable Daily Intake (ADI) value has been stated to be 10mg/kg. In this evaluation it has been stated that parabens do not accumulate in the tissues and that they are metabolized rapidly by breaking the ester bond [30].

The oestrogenic activity of parabens has first been reported for mice by Routledge et al. [31]. Afterwards, it has been stated by relevant *in vitro* studies regarding the oestrogen activity of parabens that they bond to the oestrogen receptors and activate the genes controlled by these receptors [32, 33]. However, other studies carried out have put forth that the activity of all paraben types is lower for about 1.000 to 1.000.000 times of the activity of natural oestrogen of 17β-estradiol [34]. In addition, it has also been concluded in many studies that the estrogenic activity of the parabens is not hazardous to human health [35, 36].

In a study focusing on the effects of methylparaben on the development and egg yield of *D. melanogaster*, it has been shown that 2% methylparaben concentration has displayed toxic effect and significantly decreased the number of eggs, larvae, pupa and the number of individuals that can mature, it has also been emphasized in the same study that in contrast to these results methylparaben shows estrogenic activity at a low concentration of 0.02 % and increased these ratios [37].

As a result of their acute and chronic toxicity studies carried out on mice, rats and dogs, Matthews et al. [5] have stated that parabens display a small amount of toxicity, although that they can be used safely as a food preservative.

Andersen [38] has stated that even though ethylparaben and methylparaben increases chromosomal aberrations in Chinese hamster ovary cells that they are not mutagenic. The same author has also emphasised that parabens have no effect on people other than those with allergic susceptibility.

As a result of studies carried out by Aubert et al. [39] on Sprague-Dawley rats, it has been concluded via oral, topical and subcutaneous applications that methylparaben, propylparaben and butylparaben do not accumulate enough plasma to have damaging effects on mammal organisms, that their absorption is quite good and that they break up into completely harmless small metabolites.

Other studies carried out using propylparaben and butylparaben have put forth that they may cause negative reproductive effects such as low average epididymis, seminal vesicle weights, low sperm production, low testosterone levels in young male rats [40]. However, studies carried out on other experimental animals with proper doses have put forth that parabens have no negative effects on reproductive organs [41, 42].

Parabens are thought to be a common defence in plants against bacterial or fungus infections. For instance, 800µg/gr paraben has been determined in carrot roots [43]. In addition, it has also been put forth that parabens occur naturally in bacteria, bugs, royal jelly and the vaginal fluid of female dogs [44]. In plants, it has been stated that p-hydroxybenzoic acid and its derivatives are found in plants such as barley, strawberry, red grapes, peach, carrot, onion and mango [45]. As can be understood from here, billions of humans are subject to parabens every day by eating these vegetables and fruits.

CONCLUSION

In this study, it has been determined that ethylparaben and butylparaben which are used as preservative additives in the food, drug and cosmetic industries have no genotoxic effects if used according to predetermined doses. However, when the amount of additive substances that enter our bodies every day with the food we eat is considered, care should be exercised against additives and we should at least know the content of the food we consume.

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