TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2013) 37: 683-688

doi:10.3906/biy-1303-60

Genotoxic and safety assessment of 2 parabens in somatic cells of in vivo *Drosophila melanogaster*

Arif AYAR[1], **Handan UYSAL**[2,*]

[1]Sabuncuoğlu Şerefeddin Vocational School of Health Services, Amasya University, Amasya, Turkey
[2]Department of Biology, Faculty of Science, Atatürk University, Erzurum, Turkey

Received: 26.03.2013 • **Accepted:** 20.05.2013 • **Published Online:** 08.10.2013 • **Printed:** 04.11.2013

Abstract: The objective of this study was to determine the possible genotoxic effects of the para-hydroxybenzoic acid esters (parabens) of methylparaben, propylparaben, and the mixed paraben groups formed by a combination of these, which are used as preservative substances in the food, cosmetic, and drug industries, on *Drosophila melanogaster* using the wing somatic mutation and recombination test (SMART). According to the data obtained from microscopic analysis, no increase was determined in the single type clone number at various concentrations (100, 150, 200, and 250 mM) of paraben groups in comparison with the control group. In addition, increases corresponding to the concentration increase in the single type and twin clone numbers were observed in the mixed paraben application group in comparison to the control group. However, this increase was not determined to create a statistically significant difference ($P > 0.05$). As a result of the data that we obtained, it has been concluded that parabens show toxic effects but no genotoxic effects on *D. melanogaster*.

Key words: *Drosophila melanogaster*, genotoxicity, methylparaben, propylparaben, somatic mutation, wing spot test

1. 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 the increasing of the shelf life of food products have made it obligatory to use food additives (FAs) (Ertuğrul, 1998).

Protective FAs are defined as chemical substances that protect food products from deterioration caused by various microorganisms, thus increasing their shelf lives (Parlak, 2007). To this end, many FAs are used frequently in the food industry. One of these food additive groups is the parabens. The term "paraben" is an abbreviation for para-hydroxybenzoic acid. Parabens are a family of alkyl esters of para-hydroxybenzoic acid that differ at the para position of the benzene ring by various chemical substitutions (Sasseville, 2004). The chemical substitutions provide each paraben ester with a different solubility and spectrum of antimicrobial activity. As the alkyl chain length increases, water solubility decreases and oil solubility increases (Cashman and Warshaw, 2005)

Parabens are a group of chemicals that are widely used as preserving additive substances in the food, cosmetic, and drug industries (Calafat et al., 2010). The most widely used parabens are methylparaben, ethylparaben, propylparaben, and butylparaben. Parabens are frequently used in bakery products (cakes, bread crust, fillers, etc.), drinks, fish products, aroma extracts, fruit products, gelatin, jam, gel, malt extracts, olives, pickles, salad sauces, syrups, and wine (Soni et al., 2002; CIR, 2008). Discussion regarding the safety of parabens has been going on for years within the scientific community. Many studies have put forth results stating that parabens are not toxic and that they can be used safely (Soni et al., 2005). However, with the detection of paraben traces in some breast tumors (Darbre et al., 2004) and various news items in the media regarding evidence that parabens are hazardous to human health, issues regarding the reliability of these substances have resurfaced.

Today, it is now known whether many FAs including parabens have toxic effects or not and yet these substances continue to be used recklessly. One of the most widely used genotoxicity tests carried out on insects is the wing somatic mutation and recombination test (SMART) carried out with *Drosophila* (Demir et al., 2013). SMART enables the determination of the genetic results of various chromosome aberrations such as point mutation, deletion, translocation, somatic recombination, and chromosome loss or nondisjunction (Graf et al., 1984).

* Correspondence: hauysal@atauni.edu.tr

The objective of this study was to determine the possible genotoxic effects of the parabens methylparaben, propylparaben, and mixed paraben groups used as preservatives in the food, cosmetic, and drug industries on *D. melanogaster* through SMART.

2. Materials and methods

2.1. Chemicals

Methylparaben (99.0% purity, CAS No. 99-76-3), propylparaben (99.0% purity, CAS No. 94-13-3), ethyl methanesulfonate (EMS; 100% purity, CAS no. 62-50-0), and ethyl alcohol (99.5% purity, CAS No. 64-17-5) were obtained from Sigma-Aldrich (St Louis, MO, USA), while *Drosophila* instant medium was obtained from Carolina Biological Supply (Burlington, NC, USA).

2.2. Strains

In our study, *mwh* (*mwh/mwh*) and *flr*^{3} (*flr*^{3}/*In* (*3LR*) *TM3*, *Bd*^{S}) mutant strains of *Drosophila* were used. These mutant strains carry determinant genes. Of these determinant genes, the *flare* (*flr*^{3}, 3–38.8) gene forms dulled, point-like hairs 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 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 hairs to emerge as 3 or more from the same cell (Graf et al., 1984).

2.3. Treatment procedure

LD_{50} concentrations of parabens were determined by carrying out preliminary studies. For this purpose, small culture vials were prepared with 1.5 g of dry instant *Drosophila* medium and 5 mL of the respective test solutions. A total of 100 larvae were embedded in this medium. The larvae were fed with different concentrations (100, 150, 250, 300, and 500 mM) of the parabens. Feeding ended with pupation of the surviving larvae. The experiments were repeated 3 times for each group. The results of LD_{50} concentrations were determined as 300 mM for methylparaben and propylparaben and 225 mM for the mixed paraben group. The application doses were selected to be lower than the determined LD_{50} concentrations. Afterwards, *flr*^{3} virgin females and *mwh* males of mutant strains were crossbred, and eggs were collected in periods of 8 h. The transheterozygous larvae obtained from these eggs after 72 ± 4 h were placed in application tubes containing 4 different concentrations (100, 150, 200, and 250 mM) 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 light microscope (400×) by separating them 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. Along with the experimental groups including paraben, positive control (1 mM EMS) and negative control (distilled water) groups were also prepared.

2.4. Statistical analysis

For statistical calculations, the conditional binomial test according to Kastenbaum and Bowman (1970) was used with 5% significance levels. Statistical comparisons of survival rates were made by using the chi-square test for ratios for independent samples. The differences between groups were considered significant at $P < 0.05$ and $P < 0.001$.

3. Results

When the methylparaben, propylparaben, and mixed paraben application groups were compared with the control group, no genotoxic effects were observed in our study (Tables 1 and 2). While a total of 80 wings were examined for each application group, each with normal wing (*mwh/flr*^{3}) and serrate wing (*mwh/TM3*) phenotypes, wing examination could not be carried out for the 250 mM concentration of the mixed paraben group since no living specimens could be obtained (Tables 1 and 2).

As can be seen in Tables 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 were examined, it was observed that the results were similar to those of the distilled water control group.

When Tables 1 and 2 were examined, no increase was observed in the small single type clone numbers for all application groups. Even though large single type clone and twin clone numbers increased with concentration, especially in the propylparaben and mixed paraben groups, this ratio was determined to be statistically insignificant ($P > 0.05$).

In line with the increase in concentration, the clone induction frequencies (CIFs) for the normal wing phenotype of the methylparaben application groups were 0.26, 0.26, 0.51, and 0.72 (Table 1), respectively; these ratios for the serrate phenotype were 0.31, 0.41, 0.56, and 0.61, respectively (Table 2). The CIF values for the normal wing phenotype in the propylparaben application group were 0.51, 0.56, 0.82, and 0.87 (Table 1), respectively, whereas for the serrate wing phenotype the ratios were 0.46, 0.51, 0.61, and 0.72 (Table 2), respectively. For the mixed paraben application group, these ratios were determined as 0.61, 0.82, and 1.02 for the normal wing phenotype and 0.61, 0.61, and 0.77 for the serrate wing phenotype. CIF values for the distilled water negative control group were

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 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	
Control	80	12	(0.15)		1	(0.01)		1	(0.01)		14	(0.18)		14	(0.18)		0.72
1 EMS	80	49	(0.61)	+	30	(0.38)	+	19	(0.24)	+	84	(1.05)	+	98	(1.23)	+	4.30
Methylparaben																	
100	80	4	(0.05)	-	1	(0.01)	i	0	(0.00)	i	5	(0.06)	-	5	(0.06)	-	0.26
150	80	4	(0.05)	-	1	(0.01)	i	0	(0.00)	i	5	(0.06)	-	5	(0.06)	-	0.26
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)	-	3	(0.04)	i	0	(0.00)	i	14	(0.18)	-	14	(0.18)	-	0.72
Propylparaben																	
100	80	9	(0.11)	-	2	(0.03)	i	0	(0.00)	i	10	(0.13)	-	11	(0.14)	-	0.51
150	80	8	(0.10)	-	4	(0.05)	i	0	(0.00)	i	11	(0.14)	-	12	(0.15)	-	0.56
200	80	12	(0.15)	i	5	(0.06)	i	1	(0.01)	i	16	(0.20)	i	18	(0.22)	i	0.82
250	80	11	(0.14)		5	(0.06)	i	2	(0.03)	i	17	(0.21)	i	18	(0.22)	i	0.87
Mixed parabens																	
100	80	11	(0.14)	-	1	(0.02)	i	0	(0.00)	-	12	(0.15)	-	12	(0.15)	-	0.61
150	80	12	(0.15)	i	3	(0.04)	i	1	(0.01)	-	16	(0.20)	i	16	(0.20)	i	0.82
200	80	12	(0.15)	i	5	(0.06)	i	3	(0.04)	i	20	(0.25)	i	20	(0.25)	i	1.02

No: Number of clones; Fr: frequency; D: the multiple decision procedure proposed by Frei and Würgler (1988, 1995) was applied to judge the overall response of an agent as (+) positive, (-) negative, or (i) inconclusive in the statistical analysis section; m: multiplication factor; probability levels $\alpha = \beta = 0.05$, CIF: frequency of clone formation per 10^5 cells.

determined as 0.72 for normal wing and as 0.51 for serrate wing.

When the results were compared statistically, while all results were negative for the methylparaben application group, the results for the low concentrations (100 mM and 150 mM) of the propylparaben group were negative and the results for the high concentrations (200 mM and 250 mM) were evaluated as insignificant ($P > 0.05$). Even though the results obtained for the mixed paraben application group were similar to those of the propylparaben group, the CIF value increased dramatically at the 200 mM concentration. However, this increase was determined to be insignificant.

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

The results show that the lowest survival rate was in the mixed paraben application group (Table 3). When the natural food dyes that we used in our study were compared, it was determined that the order of toxicity was methylparaben < propylparaben < mixed parabens (Table 3).

4. Discussion

It is still debated in the scientific world whether the many additives used in the food industry have toxic effects or not. When the number of substances applied to foods and the number of people subjected to them are considered, the importance of this issue can be clearly understood.

It has been determined that preservative FAs such as sodium nitrate, potassium nitrite, and potassium nitrate decrease the average life span of *D. melanogaster* at 75 mM concentrations (Sarıkaya et al., 2006). In another study examining the genotoxic effects of the same substances with SMART, it was determined that all application groups displayed genotoxic effects at 50, 75, and 100 mM concentrations, whereas the groups obtained from a mixture of these substances displayed genotoxic effects at a 25 mM concentration (Sarıkaya and Çakır, 2005).

In the study carried out by Schlatter et al. (1992) in which they examined the possible genotoxic effects via SMART of the food-preserving substances potassium sorbate, sodium sorbate, and 4,5-epoxy-2-hexenoic acid, they determined that only 4,5-epoxy-2-hexenoic acid had a weak genotoxic effect and that potassium sorbate and sodium sorbate displayed no genotoxic effects.

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
Methylparaben																	
100	80	6	(0.08)	-	0	(0.00)	i				6	(0.08)	-	6	(0.08)	-	0.31
150	80	8	(0.10)	i	0	(0.00)	i				8	(0.10)	-	8	(0.10)	-	0.41
200	80	10	(0.13)	i	1	(0.01)	i				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
Propylparaben								Balancer chromosome *TM3* does not carry the *flr*^{3} mutation									
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	2	(0.03)	i				12	(0.15)	i	12	(0.15)	i	0.61
250	80	12	(0.15)	i	2	(0.03)	i				14	(0.18)	i	14	(0.18)	i	0.72
Mixed parabens																	
100	80	11	(0.14)	-	1	(0.01)	i				12	(0.15)	i	12	(0.15)	i	0.61
150	80	11	(0.14)	-	1	(0.01)	i				12	(0.15)	i	12	(0.15)	i	0.61
200	80	12	(0.15)	i	3	(0.04)	i				15	(0.19)	i	15	(0.19)	i	0.77

No: Number of clones; Fr: frequency; D: the multiple decision procedure proposed by Frei and Würgler (1988, 1995) was applied to judge the overall response of an agent as (+) positive, (-) negative, or (i) inconclusive in the statistical analysis section; m: multiplication factor; probability levels $\alpha = \beta = 0.05$, CIF: frequency of clone formation per 10^5 cells.

Following the detection of parabens in human breast cancer tissue, their relationship with cancer has been the subject of intensive studies. Recent studies have shown their effect on the increase in the incidence of breast cancer, their preventive effects on human reproductive functions, and their estrogenic stimulus in malignant melanoma (Darbre and Harvey, 2008; Martin et al., 2010). All of these results have brought up some anxieties regarding the safe use of parabens as antimicrobial preservatives.

The estrogenic activity of parabens was first reported for mice by Routledge et al. (1998). It has since been stated in relevant in vitro studies regarding the estrogen activity of parabens that they bond to the estrogen receptors and activate the genes controlled by these receptors (Byford et al., 2002). However, other studies carried out have put forth that the activity of all paraben types is 1000 to 1,000,000 times lower than the activity of the natural estrogen 17β-estradiol (Van Meeuwen et al., 2008). In addition, it has also been concluded in many studies that the estrogenic activity of parabens is not hazardous to human health (Witorsch and Thomas, 2010; Scialli, 2011).

In a study focusing on the effects of methylparaben on the development and egg yield of *D. melanogaster*, it was shown that a 2% methylparaben concentration displayed toxic effects and significantly decreased the number of eggs, larvae, pupa, and individuals that could mature; it was also emphasized in the same study that in contrast to these results, methylparaben showed estrogenic activity at a low concentration of 0.02% and increased these ratios (Wei, 2009).

The Cosmetic Ingredient Review Expert Panel (CIR, 2008) stated that even though ethylparaben and methylparaben increase chromosomal aberrations in Chinese hamster ovary cells, they are not mutagenic. The same author also emphasized that parabens have no effect on people other than those with allergic systems.

As a result of studies carried out by Aubert et al. (2012) on Sprague–Dawley rats, it was concluded via oral, topical, and subcutaneous applications that methylparaben, propylparaben, and butylparaben do not accumulate enough plasma to have damaging effects on mammalian organisms, that their absorption is quite good, and that they break up into completely harmless small metabolites.

It has also been put forth that parabens occur naturally in bacteria, insects, royal jelly, and the vaginal fluid of female dogs. In addition, it has been stated that paraben and its derivatives are found in plants such as barley, strawberry, red grapes, peach, carrot, onion, and mango

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

Compounds	Concentration (mM)	Survival (%)	P-value
Control	Distilled water	98	-
Methylparaben	100	95	-
	150	91	-
	200	65	<0.05*
	250	52	<0.001***
Propylparaben	100	91	-
	150	88	<0.05*
	200	52	<0.05*
	250	45	<0.001***
Mixed parabens	100	68	<0.05*
	150	56	<0.05*
	200	40	<0.001***
	250	0	<0.001***

*: $P < 0.05$, survival statistics, and ***: $P < 0.001$, survival statistics (chi-square test).

(Godfrey, 2010). Therefore, billions of humans are exposed to parabens every day by eating these vegetables and fruits.

In this study, it was determined that parabens that 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 number of additive substances that enter our bodies every day with the food we eat is considered, care should be exercised with additives, and we should at least know the contents of the food we consume.

Acknowledgment

This study was supported by the Atatürk University Research Foundation (Project Code: 2010/45). The authors would like to thank Atatürk University for financial support for the project.

References

Aubert N, Ameller T, Legrand JJ (2012). Systemic exposure to parabens: pharmacokinetics, tissue distribution, excretion balance, and plasma metabolites of [14C]-methyl-, propyl- and butylparaben in rats after oral, topical or subcutaneous administration. Food Chem Toxicol 50: 445–454.

Byford JR, Shaw LE, Drew MGB, Pope GS, Sauer MJ, Darbre PD (2002). Oestrogenic activity of parabens in MCF7 human breast cancer cells. J Steroid Biochem 80: 49–60.

Calafat AM, Ye X, Wong LY, Bishop AM, Needham LL (2010). Urinary concentrations of four parabens in the U.S. population: 2005-2006. Environ Health Persp 118: 679–685.

Cashman AL, Warshaw EM (2005). Parabens: a review of epidemiology, structure, allergenicity, and hormonal properties. Dermatitis 16: 57–66.

CIR (Cosmetic Ingredient Review Expert Panel) (2008). Final amended report on the safety assessment of methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben, and benzylparaben as used in cosmetic products. Int J Toxicol 27: 1–82.

Darbre PD, Aljarrah A, Miller WR, Coldham NG, Sauer MJ, Pope GS (2004). Concentrations of parabens in human breast tumours. J Appl Toxicol 24: 5–13.

Darbre PD, Harvey PW (2008). Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase, and human exposure, and discussion of potential human health risks. J Appl Toxicol 28: 561–578.

Demir E, Kaya B, Marcos R, Kocaoğlu Cenkci S, Çetin H (2013). Investigation of the genotoxic and antigenotoxic properties of essential oils obtained from two *Origanum* species by *Drosophila* wing SMART assay. Turk J Biol 37: 129–138.

Ertuğrul N (1998). Food additives regulations and health problems about upper limit of some food additives. PhD, İstanbul University, İstanbul, Turkey.

Frei H, Würgler FE (1988). Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative, or inconclusive result. Mutat Res 203: 297–308.

Frei H, Würgler FE (1995). Optimal experimental design and sample size for the statistical evaluation of data from somatic mutation and recombination tests (SMART) in *Drosophila*. Mutat Res 334: 247–258.

Godfrey D (2010). Parabens: myth and reality. Cosmet Toiletries 125: 80–83.

Graf U, Würgler FE, Katz AJ, Frei H, Juon H, Hall CB, Kale PG (1984). Somatic mutation test in *Drosophila melanogaster*. Environ Mol Mutagen 6: 153–188.

Kastenbaum MA, Bowman KO (1970). Tables for determining the statistical significance of mutation frequencies. Mutat Res 9: 527–549.

Martin JM, Peropadre A, Herrero O, Freire PF, Labrador V, Hazen MJ (2010). Oxidative DNA damage contributes to the toxic activity of propylparaben in mammalian cells. Mutat Res 702: 86–91.

Parlak Ş (2007). The effects of food protector biphenyl on sister chromatid exchange, chromosome aberration, and micronucleus in human lymphocytes. MSc, Çukurova University, Adana, Turkey.

Routledge EJ, Parker J, Odum J, Ashby J, Sumpter J (1998). Some alkyl hydroxyl benzoate preservatives (parabens) are estrogenic. Toxicol Appl Pharm 153: 12–19.

Sarıkaya R, Çakır Ş (2005). Genotoxicity testing of four food preservatives and their combinations in the *Drosophila* wing spot test. Environ Toxicol Phar 20: 424–430.

Sarıkaya R, Çakır Ş, Solak K (2006). Effects of food preservatives on the longevity of *Drosophila melanogaster* (*mwhxflr*). Kastamonu Educ J 14: 173–184.

Sasseville D (2004). Hypersensitivity to preservatives. Dermatol Ther 17: 251–263.

Schlatter J, Würgler FE, Kranzlin R, Maier P, Holliger E, Graf U (1992). The potential genotoxicity of sorbates: effects on cell cycle in vitro in V79 cells and somatic mutations in *Drosophila*. Food Chem Toxicol 30: 843–851.

Scialli AR (2011). Reproductive effects of the parabens. Reprod Toxicol 32:138-140.

Soni MG, Carabin IG, Burdock GA (2005). Safety assessment of esters of p-hydroxybenzoic acid (parabens). Food Chem Toxicol 43: 985–1015.

Soni MG, Taylor SL, Greenberg NA, Burdock GA (2002). Evaluation of the health aspects of methyl paraben: a review of the published literature. Food Chem Toxicol 40: 1335–1373.

Van Meeuwen JA, van Son O, Piersma AH, de Jong PC, van den Berg M (2008). Aromatase inhibiting and combined estrogenic effects of parabens and estrogenic effects of other additives in cosmetics. Toxicol Appl Pharm 230: 372–382.

Wei GU (2009). Toxicity and estrogen effects of methyl paraben on *Drosophila melanogaster*. Food Sci 30: 252–254.

Witorsch RJ, Thomas JA (2010). Personal care products and endocrine disruption: a critical review of the literature. Crit Rev Toxicol 3: 1–30.