Genotoxic evaluation of bupivacaine and levobupivacaine in the *Drosophila* wing spot test

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Abstract Bupivacaine and levobupivacaine are amino amide local anesthetics commonly used in medical practice. Although bupivacaine consists of a racemic mixture of S (–)-bupivacaine and R (+)-bupivacaine enantiomers, levobupivacaine is comprised of pure S (–)-bupivacaine. It has been known that levobupivacaine is preferable to bupivacaine since it may cause cardiovascular and nervous system toxicity. For determining genotoxicity of these anesthetics, we used the wing somatic mutation and recombination test in *Drosophila melanogaster*. Three-day-old trans-heterozygous larvae were treated with bupivacaine and levobupivacaine. Analysis of the standard crosses indicated that bupivacaine and levobupivacaine did not exhibit mutagenic or recombinogenic activity until toxic doses have been reached at the larval stage. When we examined bupivacaine and levobupivacaine in the HB cross, bupivacaine did not exhibit any genotoxicity at high concentrations (500 µg/mL), but levobupivacaine did exert genotoxicity at high concentrations (1000 µg/mL)—depending on the substantial recombinogenic effect.

Keywords *Drosophila melanogaster* · Wing spot test · Bupivacaine · Levobupivacaine · Anesthesia · Genetic toxicology

Introduction

Bupivacaine is a long acting amide local anesthetic synthesized by Af Ekenstam et al. (1957); it is still most commonly used in medicinal fields, especially in surgery and obstetrics. Bupivacaine and levobupivacaine are members of the n-alkyl-substituted pipercholyxylidene family. As with all other local anesthetics, bupivacaine and levobupivacaine act by inhibition of voltage-gated sodium ion channels (Drasner 2011). Bupivacaine is a racemic mixture of two enantiomers, i.e. S (–)-bupivacaine and R (+)-bupivacaine in equal proportions. Studies have shown that S (–) or levo-bupivacaine is more reliable than R (+)-bupivacaine. Levobupivacaine is formed as the pure S (–)-bupivacaine enantiomer (Burlacu and Buggy 2008; Leone et al. 2008). After it was reported that two local anesthetic agents—etidocaine and bupivacaine—induced cardiac arrest following regional anesthesia, many studies have been done in order to determine cardiotoxic properties of local anesthetics, especially bupivacaine (Albright 1979). Bupivacaine, compared with levobupivacaine and ropivacaine, induces severe cardiovascular and central nervous system (CNS) toxicity (Huang et al. 1998; Scott et al. 1989). Due to severe toxic effects of bupivacaine, two long acting local anesthetics with a similar structure, i.e. levobupivacaine and ropivacaine, have been produced and are now available as an alternative to bupivacaine (Leone et al. 2008).

Many in vivo and in vitro test systems are currently used in genetic toxicology to determine whether any

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substance induces gene mutations or alterations in structure and number of chromosomes (Barile 2008). The somatic mutation and recombination test (SMART) is one such system that has frequently been applied to assess mutagenic and recombinogenic potentials of compounds. This test is based on the separation of imaginal discs in the embryonic stage. Imaginal discs multiply mitotically during larval development, giving rise to various structures of the adult fly following metamorphosis (Graf et al. 1998). SMART consists of the eye spot test and the wing spot test. The wing spot test (wing SMART) has the great advantage of being quick and inexpensive while providing reliability and the ability to detect genotoxic damage in one fly generation. This test method, as an *in vivo* test, may be viewed as link between microorganismal *in vitro* and mammalian *in vivo* tests (Frei and Würzler 1996). Two crossings comprise the wing spot test: (1) Standard (ST) cross and (2) High-bioactivation (HB) cross. The standard (ST) cross is used to detect the genotoxicity of any substance. The high-bioactivation (HB) cross, which is characterized by a high level of cytochrome P-450-dependent bioactivation capacity, is used to determine the genotoxicity of the degradation products of any substance (Hällström and Blanck 1984; Pacella 1993).

Initially, two new tester strains were constructed by Frölich and Würzler (1989) where these carrier chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line (ORR) (Dapkus and Merrell 1977) were used in the high-bioactivation cross. However, ORR strains present some disadvantages, such as the formation of an irregular whorling pattern in wing hairs, which makes clone classification difficult (Graf et al. 1991; Pacella et al. 1996). The gene responsible for this whorling formation is named paisley (*ply*) which is recessive and localized around position 90 of chromosome 2. This gene is linked to the *RI* gene responsible for high-bioactivation (Pacella et al. 1996). To overcome this problem, the new tester strains NORR (new ORR) was constructed by Pacella (1993). Many studies have been conducted using NORR strains (Idoamar et al. 2002; Karadeniz et al. 2011; Kaya et al. 2004; Osaba et al. 1999). NORR strains similar to ORR strains were used to screen the genotoxicity of procarcinogens and promutagens, however NORR strains are homozygous for the *RI* gene but lack the *ply* gene. This is due to separating the *ply* gene from the *RI* gene. Additionally, NORR

strains possess relatively more P450-dependent bioactivation capacity compared to ORR strains (Pacella et al. 1996).

In studies conducted in recent years, SMART seems to be an effective test system for the determination of the potential genotoxic properties of pharmaceutical agents (Gürbüzel et al. 2012, 2014; Koksall and Gürbüzel, 2015). When reviewing the literature, almost no studies were found involving an examination of the genotoxic effects of bupivacaine and levobupivacaine, even though both anesthetic agents are now commonly used in medicine. The aim of the present study was to investigate the genotoxicity of these two anesthetic agents with *Drosophila* wing SMART.

Materials and methods

Chemicals

The genotoxic effects of bupivacaine (CAS: 14252-80-3, Marcaine[®], Astra Zeneca, Istanbul, Turkey) and levobupivacaine (CAS 27262-48-2, Chirocaine[®], Abbott, Istanbul, Turkey) were tested. Before starting the study, we assessed the toxicity concentrations of the two agents using the standard LD50 test. We were able to test concentrations as high as 500 µg/mL for bupivacaine and 1000 µg/mL for levobupivacaine (data not shown). Chloral hydrate, dimethyl sulfoxide (DMSO), glycerol and gum arabic was obtained from Sigma (St. Louis, MO, USA). Bupivacaine was dissolved in DMSO and levobupivacaine was dissolved in distilled water. Thus DMSO served as a negative control for bupivacaine, distilled water served as a negative control for levobupivacaine. *Drosophila* Instant Medium (Formula 4–24) was purchased from Carolina Biological Supply (Burlington, NC, USA).

Strains

We used ST and HB strains to determine whether or not these anesthetic agents have genotoxic properties. The strains were obtained from Prof. Bülent Kaya (Akdeniz University—Turkey) and Prof. Rabia Sarıkaya (Gazi University—Turkey). Detailed information on the genetic markers and symbols used in this study can be obtained from Lindsey and Zimm (1992). Two crosses of *Drosophila* stocks were used: (1)

Standard (ST) cross: virgin females of the *mwh/mwh* crossed with *flr³/In (3LR)*, *TM3 Bd^S* males. (2) High-bioactivation (HB) cross: Virgin females of the *NORR/NORR; mwh/mwh* crossed with *NORR/NORR; flr³/In (3LR)*, *TM3 Bd^S* males.

Treatments

All study preparations were maintained at 25 ± 1 °C and a relative humidity of approximately 60 %. Eggs obtained by crossing were collected in 8 h periods in culture bottles containing a standard medium. Third-instar larvae (72 ± 4 -h old) were washed out of these bottles with tap water and placed in glass tubes containing 4.5 g of *Drosophila* instant medium and 7 mL of the respective test solutions. The larvae were fed with this medium, which contained different concentrations of bupivacaine (10, 100, 250 and 500 µg/mL) and levobupivacaine (100, 250, 500 and 1000 µg/mL). The larvae were fed in tubes until they had reached the pupal stage (48 h). Emerging adults were collected regardless of their sex, and stored in 70 % ethanol solution at +4 °C. In order to observe the mutant clones, wings were removed using pincers and mounted on microscopic slides in Faure's solution (chloral hydrate 30 g, glycerol 30 mL, gum arabic 50 mg, distilled water 50 mL). Both the dorsal and ventral surfaces of the wings were scanned at 400× magnification to determine the presence of spots.

Analysis of mutant clones

Mutant clones in the wings were classified as one of three categories: (1) small single spots consisting of one or two *mwh* cells, (2) large single spots consisting of three or more of *mwh* or *flr³* cells, and (3) twin spots consisting of a *mwh* clone adjacent to *flr³* clone. Small single spots were formed only by *mwh* cells. The recessive markers, *mwh* and *flr³* were located on the left arm of the third chromosome at map position 0.3 and 38.8, respectively, while the centromere was situated in position 47.7. The *mwh* gene is associated with multiple wing hairs instead of a single cell trichome, as in the wild-type (Frei and Würgler 1996). Sometimes, two hairs per cell may be seen in trans-heterozygous wings. These were not added to the counts of *mwh* single spots or twin spots. But, if two hairs per cell were seen in any *mwh* spot, these were taken into account in determining the size of the mutant clone (Graf et al. 1984).

The *flr³* gene generates different amorphic types of trichome (Frei and Würgler 1996). In addition, it has been known that all three mutant alleles of *flr³* show lethal effects at the embryonic stage. However, homozygous cells of the imaginal disc in terms of the *flr³* gene may be seen in the wing. Because it shows lethality in the zygote, the *flr³* allele is kept over a balancer chromosome (*TM3*) carrying multiple chromosomal inversions (Frei and Würgler 1996; Guzmán-Rincón and Graf 1995). Deletions, mitotic recombinations between *mwh* and *flr³*, non-disjunction and point mutations induce consistently the formation of single spots. Twin clones are the result of somatic recombination between the *flr³* gene and the centromere of the chromosome 3. In the *mwh/flr³* genotype (marker-heterozygous wings), the spots are induced by mitotic recombination or mutation. The only mutation induced spot formation is in the *mwh/TM3* genotype (balancer-heterozygous wings), since all the recombination events were eliminated by the *TM3* balancer chromosome (Spanó et al. 2001).

Statistical analysis

The frequency of small single spot, large single spot, twin spot, total *mwh* spots, and total spots per fly for each treatment were compared with the frequency count in the controls. The data were assessed according to “a multiple-decision procedure” described by Frei and Würgler (1988) and evaluations were classified as positive, negative or inconclusive. We used the conditional binomial test of Kastenbaum and Bowman (1970) to assess differences between the frequencies of each type of mutant clone in the treated and concurrent control flies. The clone formation frequency per 10^5 cells was computed, without size correction, based on the number of clones showing the *mwh* phenotype (i.e. *mwh* single spots and twin spots as bearing *mwh* cells zone). This frequency was obtained as the number of *mwh* clones divided by the number of wings analyzed divided by 24,400—the inspected number of cells per wing (Frei et al. 1992; Graf and Singer 1992).

Result and discussion

The results of the genotoxic evaluation of bupivacaine and levobupivacaine in the wing spot test for the ST

and HB crosses are shown in Tables 1 and 2, respectively. The tables also provide information on the two wing types for each cross, i.e. *mwh/flr³* genotype and *mwh/TM3* genotype. When looking at the tables, it can be seen that the count of total spots is derived from small single spots mostly, and only small numbers of large single spots and twin spots. All large single spots stem from *mwh* cell clones.

The series treated with bupivacaine were compared with 1 % DMSO as the concurrent negative control, and the series treated with levobupivacaine were compared with distilled water as with the concurrent negative control. It is known that spontaneous spots in the HB cross are expected to outnumber those in the ST cross (Graf and van Schaik 1992). From the data obtained in the negative controls, i.e. 1 % DMSO and distilled water, the total number of spots in the HB cross was greater than in the ST cross in the *mwh/flr³* genotypes.

Table 1 presents the results of the analyses of the wings obtained with the *mwh/flr³* genotype in ST and HB crosses. In the ST cross, bupivacaine exerted inconclusive results in terms of a genotoxic effect at all concentrations. For the HB cross, this agent exerted inconclusive or negative results at all concentrations in all spot types. These results show that bupivacaine and degradation products of bupivacaine do not induce any mutagenic or recombinogenic effects.

The results obtained from the series treated with levobupivacaine, given as *mwh/flr³* genotype in the ST and HB crosses and *mwh/TM3* genotype in HB cross, are shown in Tables 2. This agent shows inconclusive results in all spot types in the *mwh/flr³* genotype in the ST. This result indicates that levobupivacaine has no mutagenic or recombinogenic properties.

In the HB cross, all spot types in the *mwh/flr³* genotype were usually inconclusive at the three concentrations used (100, 250, and 500 µg/mL). In contrast, the highest concentration of levobupivacaine (1000 µg/mL) exerted positive effects in small single spots, total *mwh* spots, and total spots. These results demonstrate that degradation products of levobupivacaine have a genotoxic effect at the highest concentration. In the *mwh/TM3* genotype, the results are inconclusive or negative at all concentrations. When comparing the results of the *mwh/flr³* and the *mwh/TM3* genotypes in the HB cross, considering the suppression of homologous mitotic recombination in *mwh/TM3* (Spanó et al. 2001), it is plausible to assert

that the genotoxicity observed at the highest concentration stems from the substantial recombinogenic effect of levobupivacaine.

With some exceptions, the results obtained in this study are similar to those of previous studies conducted on amino amide local anesthetics. In one study, the genotoxic effects of articaine, lidocaine, and prilocaine were determined with wing SMART. When analyzing these results, prilocaine exhibited genotoxicity via the induction of homologous recombination in the ST cross. In contrast to this, the degradation products of this substance did not induce genotoxicity when employing the HB cross. However, neither lidocaine nor articaine exerted any genotoxic effects (Schneider et al. 2009). When genotoxicity was induced by repetitive administration of amino amide local anesthetics in rats, lidocaine, mepivacaine, articaine and prilocaine did not increase the frequency of micronuclei (Nai et al. 2013). Nevertheless, in another study, articaine has not shown mutagenic effects at cytotoxic concentrations in four standard in vivo and in vitro mutagenicity tests (Leuschner and Leblanc 1999). Kayukawa et al. (1988) demonstrated that lidocaine and prilocaine were negative for chromosome aberrations in the Syrian hamster embryo (SHE) cells, even when these agents were administered with an exogenous metabolic activation (Hagiwara et al. 2006).

It is known that levobupivacaine is metabolized to desbutyl levobupivacaine and 3-hydroxy levobupivacaine, respectively, via P-450 CYP3A4 and CYP1A2 enzyme isoforms in human liver. Similarly, bupivacaine is metabolized to pipercolylxylidine via P450 CYP3A4, especially, and CYP2C19 and CYP2D6 slightly in the human liver (Chalkiadis et al. 2005; Gantenbein et al. 2000; Mendola et al. 2009). One can determine the genotoxicity of degradation products of any substance with a high-bioactivation (HB) cross which is characterized by an increased cytochrome p-450-dependent bioactivation capacity. It was reported that *Drosophila melanogaster* has CYP4 and CYP6 enzyme families (Tijet et al. 2001). When degradation products of levobupivacaine induce homologous recombination, which is an errant DNA repair mechanism, it can result in loss of heterozygosity or genetic rearrangements and can lead to carcinogenesis at high concentrations i.e. 1000 µg/mL (Bishop and Schiestl 2003). We did not examine the genotoxic properties of bupivacaine at that same

Table 1 Summary of results obtained with the *Drosophila* wing SMART in the marker-heterozygous and balancer-heterozygous wings after treatment with bupivacaine: Results with the *mw/hflr*³ and *mw/h/TM3* genotypes

Test concentration ($\mu\text{g/mL}$)	Number of wings (N)	Small single spots (1–2 cells) ($m = 2$)			Large single spots (>2 cells) ($m = 5$)			Twin spots ($m = 5$)			Total <i>mw/h</i> spots ($m = 2$)			Total spots ($m = 2$)			Frequency of clone formation per 10^5 cells
		No	Fr.	D	No	Fr.	D	No	Fr.	D	No	Fr.	D	No	Fr.	D	
(A) Standard cross (ST)																	
Marker heterozygous wings (<i>mw/h/flr</i> ³)																	
1 % DMSO	50	2	0.04		0	0.00		0	0.00		2	0.04		2	0.04		0.16
10	40	5	0.13	i	0	0.00	i	0	0.00	i	5	0.13	i	5	0.13	i	0.51
100	40	5	0.13	i	0	0.00	i	0	0.00	i	5	0.13	i	5	0.13	i	0.51
250	40	6	0.15	i	0	0.00	i	0	0.00	i	6	0.15	i	6	0.15	i	0.62
500	40	4	0.10	i	2	0.05	i	0	0.00	i	6	0.15	i	6	0.15	i	0.62
(B) High-bioactivation cross (HB)																	
Marker heterozygous wings (<i>mw/h/flr</i> ³)																	
1 % DMSO	40	6	0.15		0	0.00		0	0.00		6	0.15		6	0.15		0.62
10	40	4	0.10	i	2	0.05	i	0	0.00	i	6	0.15	i	6	0.15	i	0.62
100	40	3	0.08	–	2	0.05	i	0	0.00	i	5	0.13	i	5	0.13	i	0.51
250	40	2	0.05	–	1	0.03	i	0	0.00	i	3	0.08	–	3	0.08	–	0.31
500	40	1	0.03	–	0	0.00	i	0	0.00	i	1	0.03	–	1	0.03	–	0.10

No number, Fr frequency, D statistical diagnosis according to Frei and Würigler (1988), + positive, – negative, i inconclusive, m multiplication factor, probability levels a = b = 0.05

Table 2 Summary of results obtained with the *Drosophila* wing SMART in the marker-heterozygous and balancer-heterozygous wings after treatment with levobupivacaine: results with the *mw/lf^r*³ and *mw/lf^r*³ genotypes

Test concentration ($\mu\text{g/mL}$)	Number of wings (N)	Small single spots (1–2 cells) ($m = 2$)		Large single spots (>2 cells) ($m = 5$)		Twin spots ($m = 5$)		Total <i>mw/h</i> spots ($m = 2$)		Total spots ($m = 2$)		Frequency of clone formation per 10^5 cells		
		No	Fr.	D	No	Fr.	D	No	Fr.	D	No		Fr.	D
<i>(A) Standard cross (ST)</i>														
Marker heterozygous wings (<i>mw/h</i> /f l r ³)														
Distilled water	90	9	0.10		0	0.00		0	0.00		9	0.10	0.41	
100	40	4	0.10	i	0	0.00	i	0	0.00	i	4	0.10	i	0.41
250	40	6	0.15	i	1	0.03	i	2	0.05	i	9	0.23	i	0.92
500	40	3	0.08	i	1	0.03	i	0	0.00	i	4	0.10	i	0.41
1000	40	4	0.10	i	2	0.05	i	0	0.00	i	6	0.15	i	0.62
<i>(B) High-bioactivation cross (HB)</i>														
Marker heterozygous wings (<i>mw/h</i> /f l r ³)														
Distilled water	40	5	0.13		1	0.03		0	0.00		6	0.15		0.62
100	40	14	0.35	+	0	0.00	i	0	0.00	i	14	0.35	i	1.43
250	40	8	0.20	i	2	0.05	i	0	0.00	i	10	0.25	i	1.03
500	40	10	0.25	i	0	0.00	i	0	0.00	i	10	0.25	i	1.03
1000	40	14	0.35	+	3	0.08	i	0	0.00	i	17	0.43	+	1.74
Balancer heterozygous wings (<i>mw/h</i> /T M 3)														
Distilled water	40	5	0.13		0	0.00					5	0.08		0.51
100	40	4	0.10	i	0	0.00	i			i	4	0.10	i	0.41
250	40	2	0.05	–	1	0.03	i			i	3	0.08	i	0.31
500	40	0	0.00	–	0	0.00	i			–	0	0.00	–	0.00
1000	40	0	0.00		0	0.00	i			–	0	0.00	–	0.00

No number, *Fr* frequency, *D* statistical diagnosis according to Frei and Würzler (1988), + positive, – negative, *i* inconclusive, *m*: multiplication factor, probability levels $a = b = 0.05$

concentration (1000 µg/mL) since it showed toxic effects on larval stages.

In conclusion, we have carried out genotoxicity analyses of bupivacaine and levobupivacaine and their degradation products. We have shown that bupivacaine and levobupivacaine do not exhibit genotoxic effects, a result similar to that of previous studies. In addition, while degradation products of levobupivacaine show recombinogenic effects; bupivacaine does not exhibit recombinogenic effects at a dose up to the larval toxic dose. According to our review of the literature, the present study is the first to determine the genotoxicity of bupivacaine and levobupivacaine. This study thus provides experimental data that contribute to the database on the genotoxicity of these anesthetic agents. In addition, this study shows that the *Drosophila* wing SMART is a suitable in vivo test system for evaluation of the genotoxicity of amino amide local anesthetics.

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Conflict of interest The authors declare that they have no conflict of interest.

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