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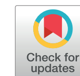
Research article

Complex interactions between nicotine and resveratrol in the *Drosophila melanogaster* wing spot test



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HIGHLIGHTS

- Nicotine was genotoxic at specific concentrations in the *Drosophila* wing spot test.
- Resveratrol protected against nicotine's genotoxic effects at some concentrations.
- Resveratrol increased nicotine's genotoxicity at specific concentrations.
- Nicotine and resveratrol have a complex interaction *in vivo*.
- Studying chemicals in combination *in vivo* may uncover unexpected interactions.

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ABSTRACT

Nicotine (NIC) and resveratrol (RES) are chemicals in tobacco and wine, respectively, that are widely consumed concurrently worldwide. NIC is an alkaloid known to be toxic, addictive and to produce oxidative stress, while RES is thought of as an antioxidant with putative health benefits. Oxidative stress can induce genotoxic damage, yet few studies have examined whether NIC is genotoxic *in vivo*. *In vitro* studies have shown that RES can ameliorate deleterious effects of NIC. However, RES has been reported to have both antioxidant and pro-oxidant effects, and an *in vivo* study reported that 0.011 mM RES was genotoxic. We used the *Drosophila melanogaster* wing spot test to determine whether NIC and RES, first individually and then in combination, were genotoxic and/or altered the cell division. We hypothesized that RES would modulate NIC's effects. NIC was genotoxic in the standard (ST) cross in a concentration-independent manner, but not genotoxic in the high bioactivation (HB) cross. RES was not genotoxic in either the ST or HB cross at the concentrations tested. We discovered a complex interaction between NIC and RES. Depending on concentration, RES was protective of NIC's genotoxic damage, RES had no interaction with NIC, or RES had an additive or synergistic effect, increasing NIC's genotoxic damage. Most NIC, RES, and NIC/RES combinations tested altered the cell division in the ST and HB crosses. Because we used the ST and HB crosses, we demonstrated that genotoxicity and cell division alterations were modulated by the xenobiotic metabolism. These results provide evidence of NIC's genotoxicity *in vivo* at specific concentrations. Moreover, NIC's genotoxicity can be modulated by its interaction with RES in a complex manner, in which their interaction can lead to either increasing NIC's damage or protecting against it.

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1. Introduction

Nicotine (NIC) is a psychoactive drug and a potent behavioral reinforcer that acts via the mesolimbic dopaminergic pathway in mammals [1]. NIC is the chemical compound responsible for tobacco addiction [2, 3] that kills more than 8 million people a year among the 1.3 billion current users worldwide [4]. Recently, NIC consumption has increased because in addition to tobacco use, there has been an increase in e-cigarette use, which delivers NIC specifically [5]. NIC is an alkaloid that is easily absorbed through the plasma membrane because of its lipophilic nature, hence it can be considered a xenobiotic. NIC is metabolized by CYP450 enzymes, which are part of the Phase I xenobiotic metabolism (XM), and by an aldehyde oxidase into secondary metabolites [6]. In mammals, the first step in the main metabolic route for NIC is catalyzed by isoforms encoded by the *CYP2A6* gene [6,7] and in *Drosophila melanogaster* variants of the XM genes *Cyp6G1* [8], *Ugt86Dd, Cyp28d1, Cyp28d2* have been associated with nicotine resistance [9,10]. NIC has detrimental effects on survival, alters brain morphology and induces behavioral changes in *Drosophila melanogaster*, which are mediated by the dopaminergic pathway, similarly to mammals [11–13]. The mechanisms that cause NIC's deleterious effects are not well understood. One hypothesis is that some of these deleterious effects are caused by NIC's ability to induce oxidative stress, genotoxicity, and cell cycle alterations. An increase in reactive oxygen species (ROS) changes the redox equilibrium, leading to oxidative stress, which can result in the oxidation of nucleic acids, which may cause mutagenesis and genome instability [14], or oxidation of proteins and lipids, which may cause loss of function and disruption of signaling pathways as those involved in cell proliferation, differentiation and apoptosis [15,16].

NIC has been shown to induce apoptosis by generating ROS, which can lead to caspase activation [17], MAPK pathway activation [18,19], or induction of pro-apoptotic agents. Chronic NIC exposure altered the cell cycle [20], affected cell viability, and induced neurotoxic damage [21]. *In vitro* studies have shown that NIC induces oxidative stress, which can lead to genotoxic damage and cell division alterations [22]. NIC was also shown to produce genotoxic damage in multiple cell lines [23–27]. However, more studies are needed to determine the genotoxic effects of NIC *in vivo*. In this work, we used the ST and HB crosses of the somatic mutation and recombination test (SMART) in *Drosophila melanogaster* wings to determine the genotoxicity of NIC *in vivo*.

Chemicals with antioxidant properties could modulate or inhibit the potential genotoxic effect of compounds that produce oxidative stress, such as NIC. Resveratrol (RES) is a polyphenol produced by many plant species widely consumed by humans, such as red wine, red grapes [28, 29]. RES absorption is close to 70%, but its bioavailability is limited because it is metabolized quickly [30] by Phase II of the XM [31]. RES has been reported to have multiple potentially beneficial effects, including having antioxidant and radical scavenging activity [32,33] and activating the base excision repair system, which may be one way it could protect against genotoxic agents [34]. RES may also exert protective effects by inhibiting CYP450 enzymes of the Phase I XM, especially CYP3A4 [35] an analog of Cyp6A2 of *D. melanogaster* [36]. *In vivo* studies have shown that RES increases the midlife-span of several model organisms, including yeast cells, worms, fruit flies, *N. furzeri* (a short-lived fish species), and obese mice [37]. Hence, RES is a known antioxidant that could modulate NIC's genotoxicity. Indeed, RES and NIC regularly co-occur in the human body of people who smoke and drink red wine. Epidemiological studies show that smoking is more prevalent in people who consume alcoholic drinks regularly, and alcoholic drinks tend to be more frequently consumed by smokers [38]. Smoking while drinking is a common behavior, hence, in such circumstances, NIC and RES enter the body concurrently and could interact *in vivo*.

While the research mentioned above suggests RES has protective effects, in the fruit fly model there are mixed results. In some studies, RES increased longevity in fruit flies in a Sir2-dependant manner [39], via increased ROS scavenging through increased antioxidant enzyme activity [40], and downregulation of genes in aging-related pathways [41]. RES also had a protective effect against toxic compounds known to induce oxidative stress including dioxins [42], 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) [43], and sodium fluoride [44]. RES also protected against ROS and cellular damage in a fruit fly model of spinocerebellar ataxia type 3 [45,46]. On the other hand, RES was not protective against paraquat-induced toxicity in a model of Parkinson's Disease in fruit flies, and induced locomotor deficits in flies [47]. In addition, RES increased the genotoxic damage induced by 4-nitroquinoline-1-oxide (4-NQO) when applied in co-treatment with this genotoxic and pro-oxidant compound in the SMART test in *D. melanogaster* [48]. RES increased the genotoxic damage of 4-NQO at a 0.011 mM concentration in flies with basal levels of Cyp450s, while only the highest concentration of RES (0.172 mM) tested had a similar effect in flies with high Cyp450s levels, which suggests that this effect of RES depends on the Cyp450 enzymes levels, and the xenobiotic metabolism [48]. It has not been tested whether 0.011 mM RES interacts with other compounds that induce oxidative damage, nor whether a lower RES concentration would also increase damage or protect instead. Hence, additional studies that determine the protective or modulatory effect of RES *in vivo* are needed.

Only a few *in vitro* studies have examined whether NIC's effects could be modulated by RES. There is evidence that RES can protect against NIC-induced oxidative damage in renal tubule cells [49] Leydig cells [50], and against NIC-induced proliferation of pancreatic cells [51]. A couple *in vivo* studies in rats showed that RES reduces NIC-induced changes in the lungs [52], and NIC-induced renal impairment after chronic NIC exposure [53]. However, the effect of RES on the putative genotoxic effects of NIC has not been studied *in vivo*.

The SMART test is a well-established *in vivo* assay used to determine the genotoxic [54] or antigenotoxic [55] potential of chemical compounds. This test also measures whether the compound causes alterations of the cell division [54]. The wing spot test determines genotoxic damage and somatic alterations *in vivo* in the standard cross (ST), with basal Cyp450s levels, and in the high bioactivation cross (HB), in which flies have high levels of these enzymes. Hence, this assay provides hints into the mechanism behind the potential damage and the XM's modulation.

In this investigation, we studied the effect of RES on the putative genotoxic and toxic NIC damage in the *in vivo* SMART wing spot test in *D. melanogaster*, using the ST and HB crosses, which have different *Cyp450s* gene expression levels. We tested *in vivo* whether RES could modulate the potential genotoxic effect of NIC. We hypothesized that there would be less damage detected in the HB cross, based on previous results [48,56]. We chose two different RES concentrations: 0.0004 mM, which we hypothesized would be protective, and 0.011 mM, which we hypothesized would increase damage.

Our results show a complex interaction between NIC and RES. Our data shows that their interaction can increase the genotoxic potential of these compounds at certain concentrations, which by themselves result in undetectable genotoxic damage. We also see examples of concentrations at which NIC by itself caused genotoxic damage, but the combination with RES showed no genotoxicity, suggesting a protective effect of RES. Whether RES was protective or increased damage varied with the RES concentration used. The lower RES concentration tested had a protective effect for NIC concentrations that caused genotoxic damage on their own. The higher RES concentration increased damage in co-treatment with NIC. Interestingly, the ST cross shows more damage than the HB cross, which reinforces the role of the XM in the effects of NIC and confirms that the XM is involved in the effects of RES.

2. Materials and methods

2.1. Chemicals

Resveratrol (RES, CAS No. 501-36-0, 99% purity), nicotine (NIC, CAS No. 54-11-5, 99% purity), ethyl carbamate (urethane: URE, CAS-No. 51-

79-6, 99% purity), were purchased from Sigma Aldrich, St. Louis MO, USA. Ethanol (EtOH, CAS No. 64-27-5, 99% purity) was bought from Merck KGaA®, Darmstadt, Germany. Aqueous solutions of NIC, EtOH, and URE were made using Milli-Q water. A 1% EtOH solution was used as co-solvent in the RES treatment. Formula 4-24 Drosophila Instant Medium (DIM) was purchased from Carolina Biological Supply Company (Burlington, NC, USA).

2.2. Strains

Drosophila melanogaster flare strain (*flr³*/In(3LR) TM3, *ri pᵖ sep l(3)89 Aa bx34e e Bdˢ*), Oregon R(R)-flare (ORR(1); ORR(2); *flr³*/In(3LR) TM3, *ri pᵖ sep l(3)89 Aa bx34e e Bdˢ*) and multiple wing hairs (*mwh/mwh*) were propagated in culture medium [48]. These strains were originally donated by Ulrich Graf of the ETH, Zurich, Switzerland. Phenotypic markers were verified continuously. For detailed features of the strains see [57,58,59,60].

2.3. Crosses

Virgin females from the flare and Oregon R(R)-flare strains were mated to multiple wing hair males to generate the standard cross (ST) and the high bioactivation cross (HB), respectively [54,61,62]. To synchronize the cultures and get third instar larvae (72 ± 4 h), an 8-h egg collection was taken for each cross at 25 °C and 65% relative humidity in a thick layer of fermenting, live, baker's yeast supplemented with sucrose. The larvae were washed out of the culture vials with tap water at room temperature (25 °C) through a fine-meshed-stainless-steel strainer [63].

2.4. Treatments

A minimum of three independent experiments per treatment were conducted with both crosses. Collected larvae (72 ± 4 h) were placed into vials with 0.5 g of DIM hydrated with 2 mL of treatment solution. Chronic treatments, until pupation, were applied at the following final concentrations: EtOH 1% (dissolvent control for RES), 20 mM URE (positive control; [64], 0.0004 mM and 0.011 mM RES in a EtOH 1% solution [34, 48,65,66]; NIC solutions were 0.0125 mM, 0.025 mM, 0.050 mM, 0.456 mM, 1.9 mM, 3.8 mM and 11.4 mM [12,13,21,23–27,67–70] prepared with Milli-Q water. The following co-treatments were tested: 0.0004 mM RES + 0.456 mM/1.9 mM/3.8 mM/11.4 mM NIC and 0.011 mM RES + 0.0125 mM/0.025 mM/0.050 mM/0.456 mM/1.9 mM/3.8 mM/11.4 mM NIC.

2.5. Drosophila wing spot test

Treatment vials containing the third instar larvae from both crosses were incubated at 25 °C and 65% humidity until the emergence of imagos, which were collected and preserved in EtOH 70%. Slides with the wings of trans-heterozygous individuals (*mwh* +/+ *flr³*) were mounted on Entellan®. An even sex proportion was attempted for every treatment scored. Slides were scored under a light microscope (400X) [54]. Scoring was carried out using a double-blind method and including 55 to 60 individuals per treatment whenever possible [71]. Statistical analyses were done with the SMART-PC vs 2.1 software (Frei and Würgler, unpublished), which is based on the Kastenbaum-Bowman test ($P < 0.05$) [72] and the non-parametric Mann-Whitney and Wilcoxon U test ($\alpha = \beta = 0.05$) [71]. Alterations in cell division caused by the treatments were compared to their respective controls through the Kolmogorov-Smirnov test ($P < 0.05$) to analyze the cumulative *mwh* clone size class distribution. This analysis takes into account the spontaneous or induced rates of *mwh* mutant clones and the size they reach through the wing imaginal disc cell divisions [54,73]. At a minimum, three independent experiments per treatment and cross were analyzed.

3. Results

First, we used the SMART wing spot test to determine *in vivo* whether NIC induced genotoxic damage and/or mitotic alterations in the imaginal wing's cells. We tested a range of NIC concentrations that have been shown to induce oxidative stress and genotoxic damage or have been tested in other *Drosophila* studies (See Methods: Treatments). Next, we used the SMART wing spot test to determine whether RES in co-treatment with NIC would modulate NIC's effects. We selected the two RES concentrations based on bioavailability data for RES [30,31] or that have been previously tested in *Drosophila* [48] (See Methods: Treatments).

Below we show the results of this investigation. The results are organized by treatment. Within each treatment, the information is organized showing examples of increased damage first, then examples with no damage. To determine cell cycle alterations we compared the *mwh* clone size class cumulative distribution results against their corresponding 1% EtOH control or against NIC or RES treatments in order to identify any interactions between them [54,73].

3.1. The EtOH dissolvent control for RES contributes to mitotic alterations in the HB cross

RES was dissolved in 1% EtOH. We first determined whether the 1% EtOH dissolvent control on its own was genotoxic. There were no differences in spot frequencies when the 1% EtOH treatment was compared with Milli-Q water dissolvent in both the ST and HB crosses (Table 1. However, we found cell division alterations in the HB cross, but not in the ST cross (Table 2).

3.2. Nicotine is genotoxic and causes mitotic alterations at specific concentrations

The NIC treatment was genotoxic at the smallest (0.0125 mM) and highest (11.4 mM) NIC concentrations tested in the ST cross. Both concentrations increased the frequency of small and total spots. In addition, the 11.4 mM NIC treatment decreased the number of ST cross's surviving flies. In the HB cross, the 0.0125 mM NIC treatment was not genotoxic, while the 11.4 mM was lethal. The NIC treatments at 0.025 mM, 0.05 mM, 0.456 mM, 1.9 mM, and 3.8 mM did not show genotoxic damage in neither the ST nor the HB cross (Tables 3, 4, and 5).

The *mwh* clone size class distribution was altered in the 0.025 mM, 0.050 mM, and 3.8 mM NIC concentrations in both crosses. The 0.0125 mM concentration affected the *mwh* clone size class distribution in the ST cross, but not in the HB cross, while the 0.456 mM NIC concentration affected the *mwh* clone size class distribution in the HB cross, but not in the ST cross. The 1.9 mM NIC concentration did not affect the *mwh* clone size class distribution in the ST, but the results were inconclusive for the HB cross. In 11.4 mM NIC, the *mwh* clone size class distribution was altered in the ST cross. It was not possible to determine the effect on the HB cross, because there were no survivors (Table 2).

These results show that NIC is genotoxic and causes mitotic alterations. However, these effects are not directly correlated with concentration. The different effects in the ST and HB crosses show that differences in the XM modulate the genotoxic effect of NIC but are not clearly involved in the changes in *mwh* clone size class distribution.

3.3. Resveratrol was not genotoxic but caused mitotic alterations

RES was reported genotoxic on the SMART test at the 0.011 mM concentration [48]. We aimed to replicate previous results and also test if a lower RES concentration, 0.0004 mM, would be protective against the putative oxidative stress of NIC. Hence, we first determined whether these two concentrations were genotoxic or altered the *mwh* clone size class distribution in our assay.

Table 1. Summary of results obtained in the standard (ST) and high bioactivation (HB) crosses of the *Drosophila* wing SMART after scoring marker-heterozygous flies (*mwh* +/+ *flr*[3], wild-type wings) treated with resveratrol (RES) at [0.0, 0.0004, 0.011 mM]; Milli-Q water and ethanol 1% as dissolvent controls.

Compound Cross[b]	Type	Number of flies	Spots per Fly (Number of Spots) Statistical Diagnosis[a]					Mean	Clone formation	
			Small single spots (1-2 cells)	large single spots (>2 cells)	Twin spots	Total spots	*mwh* clones	*mvh* clone size class	per 105 per cell observed	cells division[c] control connected
			m = 2	m = 5	m = 5	m = 5				
ST										
#1 Assay										
Dissolvent control										
Water		51	0.47 (024)	0.10 (005)	0.00 (000)	0.57 (029)	29	1.69	1.25	
EtOH	1 %	57	0.39 (022) -	0.09 (005) -	0.00 (000) -	0.47 (027) -	27	1.81	0.95	-0.25
RES treatments										
EtOH	1 %	57	0.39 (022)	0.09 (005)	0.00 (000)	0.47 (027)	27	1.81	0.95	
RES	0.0004	58	0.53 (031) -	0.07 (004) -	0.00 (000) -	0.60 (035) -	33	1.64	1.15	0.20
RES	0.0110	58	0.47 (027) -	0.03 (002) -	0.07 (004) +	0.57 (033) -	32	1.84	1.15	0.20
#2 Assay										
Dissolvent control										
Water		61	0.46 (028)	0.07 (004)	0.02 (001)	0.54 (033)	33	1.70	1.1	
EtOH	1 %	60	0.45 (027) -	0.15 (009) -	0.05 (003) -	0.65 (039) -	38	2.34	1.3	0.20
RES treatment										
EtOH	1 %	60	0.45 (027)	0.15 (009)	0.05 (003)	0.65 (039)	38	2.34	1.30	
RES	0.0110	99	0.58 (057) -	0.05 (005) -	0.04 (004) -	0.67 (066) -	65	1.58	1.35	0.05
HB										
#1 Assay										
Dissolvent control										
Water		35	0.54 (019)	0.06 (002)	0.00 (000)	0.60 (021)	21	1.29	1.25	
EtOH	1 %	35	0.34 (012) -	0.09 (003) -	0.03 (001) -	0.46 (016) -	16	1.94	0.95	-0.3
RES treatment										
EtOH	1 %	35	0.34 (012)	0.09 (003)	0.03 (001)	0.46 (016)	16	1.94	0.95	
RES	0.0004	87	0.52 (045) -	0.06 (005) -	0.01 (001) -	0.59 (051) -	51	1.84	1.20	0.25
#2 Assay										
Dissolvent control										
Water		61	0.66 (040)	0.07 (004)	0.00 (000)	0.72 (044)	44	1.43	1.5	
EtOH	1 %	60	0.60 (036) -	0.08 (005) -	0.00 (000) -	0.68 (041) -	40	1.37	1.35	-0.15
RES treatment										
EtOH	1 %	60	0.60 (036)	0.08 (005)	0.00 (000)	0.68 (041)	40	1.37	1.35	
RES	0.0110	60	0.73 (044) -	0.07 (004) -	0.02 (001) -	0.82 (049) -	48	1.56	1.65	0.25
#3 Assay										
Dissolvent control										
Water		50	0.50 (025)	0.10 (005)	0.04 (002)	0.64 (032)	32	1.66	1.30	
EtOH	1 %	60	0.33 (020) -	0.17 (010) -	0.00 (000) -	0.50 (030) -	28	2.00	0.95	-0.35
RES treatment										
EtOH	1 %	60	0.33 (020)	0.17 (010)	0.00 (000)	0.50 (030)	28	2.00	0.95	
RES	0.0110	59	0.36 (021) -	0.02 (001) ↓	0.02 (001) -	0.39 (023) -	23	1.43	0.80	-0.15

[a] Statistical diagnoses according to Frei and Würgler (1988) m: minimal risk multiplication factor for the assessment of negative results. For the final statistical diagnosis of all positive (+) and negative (-) results; (↓) significant decrease; the non-parametric Mann-Whitney and Wilcoxon U-test with significance levels α and β = 0.05 was used to exclude false positive or negative diagnoses [Frei and Würgler, 1995]. One side binomial test, significance levels α and β: significative results: + (α ≤ 0.05; no significant results: - (β ≤ 0.05).

[b] ST: standard cross; HB: high bioactivation cross.

[c] Clone frequencies per fly divided by the number of cells examined per fly (48,800) gives an estimate of formation frequencies per cell and per cell division in chronic exposure experiments [Frei and Würgler, 1995].

Table 2. Statistical comparisons between Nicotine, Resveratrol vs. dissolvent controls[a] or co-treatments[b] of the accumulated *mwh* clones size class distribution in ST and HB crosses[c].

Control	Treatment	Diagnosis	
		ST cross	HB cross
a) Distilled water[a] vs ethanol (%)			
Water	Ethanol 1	-	i
b) Distilled water[a] vs Nicotine (mM)			
Water	Nicotine 0.0125	+	-
	Nicotine 0.025	+	+
	Nicotine 0.050	+	+
	Nicotine 0.456	-	+
	Nicotine 1.9	-	i
	Nicotine 3.8	+	+
	Nicotine 11.4	+	☠
c) Ethanol[a] (%) vs Resveratrol (mM)			
Ethanol 1	Resveratrol 0.0004	+	+
	Resveratrol 0.011	+	i
d) Ethanol[a] (%) vs Nicotine (mM) + RES 0.0004 mM			
Ethanol 1	Nicotine 0.456 + RES 0.0004 mM	+	+
	Nicotine 1.9 + RES 0.0004 mM	+	-
	Nicotine 3.8 + RES 0.0004 mM	+	+
	Nicotine 11.4 + RES 0.0004 mM	+	+
e) Ethanol[a] (%) vs Nicotine (mM) + RES 0.011 mM			
Ethanol 1	Nicotine 0.0125 + RES 0.011 mM	+	+
	Nicotine 0.025 + RES 0.011 mM	+	-
	Nicotine 0.050 + RES 0.011 mM	-	-
	Nicotine 0.456 + RES 0.011 mM	+	+
	Nicotine 1.9 + RES 0.011 mM	+	-
	Nicotine 3.8 + RES 0.011 mM	+	+
	Nicotine 11.4 + RES 0.011 mM	+	☠
f) RES 0.0004 mM vs Nicotine (mM) + RES 0.0004 mM[b]			
RES 0.0004 mM	Nicotine 0.456 + RES 0.0004 mM	+	+
	Nicotine 1.9 + RES 0.0004 mM	+	+
	Nicotine 3.8 + RES 0.0004 mM	+	-
	Nicotine 11.4 + RES 0.0004 mM	+	+
g) RES 0.011 mM vs Nicotine (mM) + RES 0.011 mM[b]			
RES 0.011 mM	Nicotine 0.0125 + RES 0.011 mM	+	+
	Nicotine 0.025 + RES 0.011 mM	-	+
	Nicotine 0.050 + RES 0.011 mM	+	+
	Nicotine 0.456 + RES 0.011 mM	+	-
	Nicotine 1.9 + RES 0.011 mM	+	-
	Nicotine 3.8 + RES 0.011 mM	+	+
	Nicotine 11.4 + RES 0.011 mM	+	☠
h) Nicotine (mM) vs Nicotine (mM) + RES 0.0004 mM[b]			
Nicotine 0.456	Nicotine 0.456 + RES 0.0004 mM	+	+
Nicotine 1.9	Nicotine 1.9 + RES 0.0004 mM	+	+
Nicotine 3.8	Nicotine 3.8 + RES 0.0004 mM	+	+
Nicotine 11.4	Nicotine 11.4 + RES 0.0004 mM	+	☠
i) Nicotine (mM) vs Nicotine (mM) + RES 0.011 mM[b]			
Nicotine 0.0125	Nicotine 0.0125 + RES 0.011 mM	+	+
Nicotine 0.025	Nicotine 0.025 + RES 0.011 mM	+	+
Nicotine 0.050	Nicotine 0.050 + RES 0.011 mM	+	+
Nicotine 0.456	Nicotine 0.456 + RES 0.011 mM	+	-
Nicotine 1.9	Nicotine 1.9 + RES 0.011 mM	+	i
Nicotine 3.8	Nicotine 3.8 + RES 0.011 mM	+	+
Nicotine 11.4	Nicotine 11.4 + RES 0.011 mM	+	☠

[c]Kolmogorov–Smirnov test was performed to statistically analyze the accumulated *mwh* clones size class distribution in each treatment or co-treatment against the corresponding control ($P < 0.05$); positive (+) results indicate statistical significant alteration of the cell division on imaginal wing cells, therefore, there are cytotoxic effects (Santos-Cruz *et al.*, 2020; Graf *et al.*, 1984); negative (-) results signify that $P > 0.05$; (☠) signifies that treatment or co-treatment was lethal with no survivors; (i) signifies "inconclusive" which we use to denote cases when two or more independent tests were conducted and statistical results did not coincide.

The 0.0004 mM RES treatment was not genotoxic in either the ST or the HB crosses (Tables 1 and 2). Surprisingly, the 0.011 mM RES treatment showed no genotoxicity in either the ST or the HB cross on a first assay we conducted. Because of the discrepancy with previous results [48], we carried out a second assay and found a significant difference only in twin spot frequency in the ST cross and a small decrease in the number of large spots in the HB cross. These results correlated with the high frequency of spots in the dissolvent control (Table 1).

Surprisingly, the 0.0004 mM RES treatment altered the *mwh* clone size class distribution in both crosses (Table 2). In the ST cross, the 0.011 mM RES concentration altered it in both assays performed. However, the *mwh* clone size class distribution was modified in one assay but not the other in the HB cross (Table 2).

These results show that RES was not genotoxic at the 0.0004 mM concentration but caused cytotoxic damage. The 0.011 mM RES concentration may be genotoxic and cytotoxic or not depending on the results of the dissolvent control it is compared against, which would explain the assay-by-assay variability we encountered.

3.4. The NIC-RES co-treatments show a complex interaction between these two chemicals

We combined NIC and RES to investigate if RES modulated the effects of NIC. We hypothesized that 0.0004 mM RES in co-treatment with NIC would ameliorate the putative deleterious effects of NIC, while 0.011 mM RES in co-treatment with NIC would lead to a synergistic effect with increase damage. There were differences in the effects we detected when NIC was combined with the low (0.0004 mM) versus the high (0.011 mM) RES concentration. Interestingly, the interaction between NIC and RES differed in the ST cross and the HB cross. Also, we observed different types of NIC-RES interaction depending on the specific NIC-RES concentration combination: increased damage, decreased damage and no effect.

3.4.1. NIC + 0.0004 mM RES co-treatments

First, we tested the high NIC concentrations (0.456 mM, 1.9 mM, 3.8 mM, and 11.4 mM) in co-treatment with the lower RES concentration (0.0004 mM).

Increased genotoxic effect between NIC 1.9 mM *and 11.4* mM *and RES in the ST cross:* There was an increase in genotoxic damage (large and total spots) for the 1.9 mM and the 11.4 mM NIC concentrations + 0.0004 mM RES when comparing against the dissolvent control, NIC alone, or RES alone (Tables 3, 6, and 7). This suggests an interaction between NIC and RES that results in an unexpected synergistic effect in the ST cross for these co-treatments.

No interaction between NIC 0.456 mM *and 3.8* mM *and RES in the ST cross:* There was no interaction between 0.0004 mM RES and either 0.456 mM or 3.8 mM NIC, which were not genotoxic on their own, nor in combination with 0.0004 mM RES in the ST cross.

Increased genotoxic effects between NIC 0.456 mM *and 3.8* mM *and RES in the HB cross:* The 0.456 mM and 3.8 mM NIC concentrations were not genotoxic in combination with 0.0004 mM RES in the ST cross. In contrast, these co-treatments had an increase in spots frequencies in the HB cross (Tables 4, 6, and 7). This suggests that the XM plays a role in the NIC-RES interaction in these co-treatments.

Protective effects of RES in the HB cross at 1.9 mM *and 11.4* mM *NIC:* In the HB cross, the 1.9 mM NIC + 0.0004 mM RES co-treatment showed no genotoxicity, and there was a significant decrease in the number of small spots in comparison with 0.0004 mM RES alone (Tables 4, 6, and 7). This

Table 3. . Summary of results obtained in the standard (ST) and high bioactivation (HB) crosses of the *Drosophila* wing SMART after scoring marker-heterozygous flies (*mwh* +/+ *flr*[3], wild-type wings) treated with nicotine (NIC) at [0, 0.0125, 0.025, 0.05, 0.456, 1.9, 3.8, 11.4 mM]; urethane (URE)[20 mM] and Milli-Q water as positive and dissolvent controls, respectively.

Compound Cross[b]		Number of flies	Spots per Fly (Number of Spots) Statistical Diagnosis[a]					Mean	Clone formation	
	Type		Small single spots	large single spots	Twin spots	Total spots	*mwh*clones	*mvh* clone size class	per 105 per cell	cells division[c]
			(1-2 cells)	(>2 cells)					observed	control connected
			m = 2	m = 5	m = 5	m = 5				
ST										
Positive control										
Water		61	0.46 (28)	0.07 (04)	0.02 (1)	0.54 (33)	33	1.70	1.10	
URE	20.0	28	1.21 (34) +	0.11 (03) -	0.04 (1) -	1.36 (38) +	38	1.45	2.80	1.65
NIC treatments										
Water	0	61	0.46 (28)	0.07 (04)	0.02 (1)	0.54 (33)	33	1.70	1.10	
NIC	0.0125	44	1.16 (51) +	0.43 (19) -	0.00 (0) -	1.59 (70) +	70	2.26	3.25	2.15
NIC	0.0250	41	0.66 (27) -	0.00 (00) -	0.02 (1) -	0.68 (28) -	28	1.21	1.40	0.30
NIC	0.0500	21	0.67 (14) -	0.10 (02) -	0.05 (1) -	0.81 (17) -	17	1.47	1.65	0.55
Water	0	51	0.47 (24) -	0.10 (05)	0.00 (0)	0.57 (29)	29	1.69	1.15	
NIC	0.4560	38	0.68 (26) -	0.05 (02) -	0.00 (0) -	0.74 (28) -	28	1.54	1.50	0.35
NIC	1.9000	46	0.57 (26) -	0.04 (02) -	0.02 (1) -	0.63 (29) -	28	1.57	1.25	0.10
NIC	3.8000	32	0.62 (20) -	0.03 (01) -	0.03 (1) -	0.69 (22) -	22	1.45	1.40	0.25
NIC	11.4000	22	1.27 (28) +	0.23 (05) -	0.05 (1) -	1.55 (34) +	34	1.71	3.15	2.00
HB										
Positive control										
Water	0	61	0.66 (40)	0.07 (04)	0.00 (0)	0.72 (44)	44	1.43	1.50	
URE	20.0	23	2.52 (58) +	0.39 (09) +	0.17 (4) +	3.09 (71) +	71	1.82	6.35	4.85
NIC treatments										
Water	0	61	0.66 (40)	0.07 (04)	0.00 (0)	0.72 (44)	44	1.43	1.50	
NIC	0.0125	60	0.68 (41) -	0.05 (03) -	0.02 (1) -	0.75 (45) -	42	1.14	1.45	-0.05
NIC	0.0250	60	0.65 (39) -	0.03 (02) -	0.00 (0) -	0.68 (41) -	41	1.29	1.40	-0.10
NIC	0.0500	60	0.77 (46) -	0.08 (05) -	0.00 (0) -	0.85 (51) -	51	1.61	1.75	0.25
#1 Assay*										
Water	0	35	0.54 (19)	0.06 (02)	0.00 (0)	0.60 (21)	21	1.29	1.25	
NIC	0.4560	99	0.38 (38) -	0.07 (07) -	0.03 (3) -	0.48 (48) -	47	1.64	0.95	-0.25
NIC	1.9000	58	0.47 (27) -	0.05 (03) -	0.07 (4) -	0.59 (34) -	34	1.82	1.20	-0.05
NIC	3.8000	69	0.36 (25) -	0.06 (04) -	0.00 (0) -	0.42 (29) -	28	1.61	0.85	-0.40
#2 Assay*										
Water	0	50	0.50 (25) -	0.10 (05)	0.04 (2)	0.64 (32)	32	1.66	1.30	
NIC	0.4560	60	0.33 (20) -	0.08 (05) -	0.02 (1) -	0.43 (26) -	25	1.64	0.85	-0.45
NIC	1.9000	58	0.47 (27) -	0.05 (03) -	0.07 (4) -	0.59 (34) -	34	1.82	1.20	-0.10
NIC	3.8000	60	0.37 (22) -	0.05 (03) -	0.00 (0) -	0.42 (25) -	24	1.42	0.80	-0.50

[a] Statistical diagnoses according to Frei and Würgler (1988) m: minimal risk multiplication factor for the assessment of negative results. For the final statistical diagnosis of all positive (+) and negative (-) results; the non-parametric Mann-Whitney and Wilcoxon *U*-test with significance levels α and $\beta = 0.05$ was used to exclude false positive or negative diagnoses [Frei and Würgler, 1995]. One side binomial test, significance levels α and β: significant results: + ($\alpha \leq 0.05$; no significant results: - ($\beta \leq 0.05$).

[b] ST: standard cross; HB: high bioactivation cross.

[c] Clone frequencies per fly divided by the number of cells examined per fly (48,800) gives an estimate of formation frequencies per cell and per cell division in chronic exposure experiments [Frei and Würgler, 1995].*In HB cross the NIC [11.4 mM] was lethal.

Table 4. Genotoxic damage for ST and HB crosses with high [NIC]. Summary evaluation as not-genotoxic (-) or genotoxic (+) of each high NIC concentration treatment (NIC of 0.456 mM, 1.9 mM, 3.8 mM or 11.4 mM with no RES = 0.0 mM) and of the co-treatments (same NIC concentrations plus either 0.0004mM RES or 0.011mM RES) in the ST and the HB crosses. The co-treatment results were evaluated against the results for the ETOH dissolvent control for RES, the results for NIC (without RES) and the results for RES (without NIC). These comparisons were done with the non-parametric Mann-Whitney and Wilcoxon *U*-test $P < 0.05$ to provide additional information about which component of the co-treatment had the greatest effect.

ST cross				Control or treatment in corresponding cross	HB cross			
Nicotine (mM)	Resveratrol (mM)				Resveratrol (mM)			Nicotine (mM)
	0.0*	0.0004	0.011		0.0*	0.0004	0.011	
0.456	-	-	+	EtOH	-	+	-	0.456
		-	-	NIC		-	-	
		-	-	RES		-	-	
1.9	-	+	+	EtOH	-	-	-	1.9
		+	+	NIC		-	-	
		+	+	RES		↓	-	
3.8	-	-	+	EtOH	-	+	-	3.8
		-	-	NIC		+	+	
		-	-	RES		-	+	
11.4	+	+	-	EtOH		-	☠	11.4
		+	↓	NIC	☠	☠	☠	
		+	+	RES		-	☠	

[*] NIC was compared against Milli-Q water to determine genotoxicity. + signifies an increase with $P < 0.05$ ↓ signifies a decrease with $P < 0.05$; - signifies that $P > 0.05$, ☠ signifies that treatment was lethal with no survivors.

Table 5. Genotoxic damage for ST and HB crosses with low [NIC]. Summary evaluation as not-genotoxic (-) or genotoxic (+) of each low NIC concentration treatment (NIC of 0.0125 mM, 0.025 mM, or 0.05 mM with no RES = 0.0 mM) and of the co-treatments (same NIC concentrations plus 0.011mM RES) in the ST and the HB crosses. The co-treatment results were evaluated against the results for the ETOH dissolvent control for RES, the results for NIC (without RES) and the results for RES (without NIC). These comparisons were done with the non-parametric Mann-Whitney and Wilcoxon *U*-test $P < 0.05$ to provide additional information about which component of the co-treatment had the greatest effect.

ST cross			Control or treatment in corresponding cross	HB cross		
Nicotine (mM)	Resveratrol (mM)			Resveratrol (mM)		Nicotine (mM)
	0.0*	0.011		0.0*	0.011	
0.0125	+	+	EtOH	-	-	0.0125
		↓	NIC		-	
		-	RES		-	
0.0250	-	+	EtOH	-	-	0.0250
		+	NIC		-	
		+	RES		-	
0.0500	-	-	EtOH	-	-	0.0500

[*] NIC was compared against Milli-Q water to determine genotoxicity. + signifies an increase with $P < 0.05$; ↓ signifies a decrease with $P < 0.05$; - signifies that $P > 0.05$.

suggests an interaction between NIC and RES, in which RES is protecting against NIC's effects. The 11.4 mM NIC + 0.0004 mM RES co-treatment was not genotoxic in the HB cross, based on the frequency of spots. However, there was a decrease in lethality in this co-treatment: while the 11.4 mM NIC treatment had no survivors, the co-treatment had a few survivors (Tables 4, 6, and 7). This suggests that 0.0004 mM RES had a protective effect against the toxicity of 11.4 mM NIC in the HB cross.

Mitotic alterations in most NIC-RES co-treatments in both crosses: All co-treatments between the high NIC concentrations (0.456 mM, 1.9 mM, 3.8 mM, and 11.4 mM) and the lower RES concentration (0.0004 mM) altered the *mwh* clone size class distribution in the ST cross (Table 2). The same was true for the HB cross, except for two co-treatments in which the *mwh* clone size class distribution was not significantly different: (1) 1.9 mM NIC + 0.0004 mM RES compared against the dissolvent control; (2) 3.8 mM NIC + 0.0004 mM RES compared against 0.0004 mM RES. The *mwh* clone size class distribution could not be evaluated in the 11.4 mM NIC + 0.0004 mM RES co-treatment against the 11.4 mM NIC treatment because there were no survivors (Table 2).

These results show that NIC interacts with 0.0004 mM RES at specific NIC concentrations. Opposite to what we hypothesized, two of four the NIC concentrations tested (1.9 mM and 11.4 mM NIC) interacted with RES in the ST cross in a synergistic manner. All four NIC concentrations had an interaction with RES in the HB cross, but in addition to increases in damage, we also saw protection, which was what we had hypothesized would happen. Interestingly, the two NIC concentrations that showed damage in co-treatment in the ST cross, showed protection in the HB cross. This demonstrates a role for the XM in mediating the genotoxic effect. We saw a widespread effect of NIC on the cell division at multiple different concentrations and with only small differences between the ST and HB crosses. This suggests the XM does not modulate the effect of the 0.0004 mM RES + NICs co-treatments on mitosis.

3.4.2. NIC + 0.011 mM RES co-treatments

Given the mixed results with the 0.0004 mM RES concentration co-treatments, which included examples of synergy, protection, and lack of interaction with NIC at specific concentrations, we next tested whether a higher RES concentration (0.011 mM RES) would result in NIC-RES interaction with all the higher NIC concentrations tested (0.456 mM, 1.9 mM, 3.8 mM, and 11.4 mM). We also tested whether we could detect interaction between 0.011 mM RES and lower NIC concentrations (0.0125 mM, 0.025 mM, and 0.05 mM).

No interaction between NIC 0.05 mM and RES in the ST cross: The 0.05 mM NIC + 0.011 mM RES co-treatment was not genotoxic in the ST cross. Hence, there was no interaction between these treatments (Tables 5, 6, and 7).

Synergy between NIC 0.025 mM or 1.9 mM, and RES in the ST cross: The 0.025 mM and 1.9 mM NIC were not genotoxic on their own but became genotoxic and had increased damage in co-treatments with 0.011 mM RES compared against the dissolvent control, NIC alone, or RES alone. This suggests a synergistic effect between NIC and RES with these co-treatments in the ST cross (Tables 4, 5, 6, and 7).

Additive effect between NIC 0.456 or 3.8 mM and RES in the ST cross: The 0.456 mM and 3.8 mM NIC concentrations were not genotoxic on their own but were genotoxic compared to the dissolvent control in co-treatment with 0.011 mM RES in the ST cross. However, there was no

Table 6. Summary of results obtained in the standard (ST) and high bioactivation (HB) crosses of the *Drosophila* wing SMART after scoring marker-heterozygous flies (*mwh* +/+ *flr*[3], wild-type wings) treated with nicotine (NIC) at [0.0, 0.0125, 0.0250, 0.050, 0.456, 1.9, 3.8 and 11.4 mM] + resveratrol (RES) [0.0004 or 0.0110 mM]; ethanol (1%) as dissolvent control.

Compound Cross[b]	Type	Number of flies	Spots per Fly (Number of Spots) Statistical Diagnosis[a] Small single spots (1-2 cells) m = 2	large single spots (>2 cells) m = 5	Twin spots m = 5	Total spots m = 5	*mwh*clones	Mean *mvh* clone size class	Clone formation per 105 per cell observed	cells division[c] control connected
NIC treatments + RES [0.0004 mM]										
ST										
EtOH	1 %	57	0.39 (022)	0.09 (005)	0.00 (000)	0.47 (027)	27	1.81	0.95	
NIC+RES	0.4560	33	0.61 (020) -	0.06 (002) -	0.00 (000) -	0.67 (022) -	22	1.45	1.35	0.40
NIC+RES	1.9000	22	0.77 (017) +	0.27 (006) +	0.00 (000) -	1.05 (023) +	23	1.87	2.15	1.15
NIC+RES	3.8000	38	0.42 (016) -	0.05 (002) -	0.00 (000) -	0.47 (018) -	16	1.06	0.85	-0.10
NIC+RES	11.4000	13	0.92 (012) +	0.38 (005) +	0.00 (000) -	1.31 (017) +	17	2.47	2.70	1.70
HB										
EtOH	1%	35	0.34 (012) -	0.09 (003)	0.03 (001)	0.46 (016)	16	1.94	0.95	
NIC+RES	0.4560	64	0.36 (023) +	0.11 (007) -	0.00 (000) -	0.47 (030) -	29	2.21	0.95	0.00
NIC+RES	1.9000	49	0.29 (014) -	0.08 (004) -	0.02 (001) -	0.39 (019) -	18	2.33	0.75	-0.20
NIC+RES	3.8000	78	0.59 (046) +	0.05 (004) -	0.03 (002) -	0.67 (052) -	52	1.63	1.35	0.45
NIC+RES	11.4000	10	0.40 (004) -	0.00 (000) -	0.00 (000) -	0.40 (004) -	04	1.25	0.80	-0.10
NIC treatments + RES [0.0110 mM]										
ST										
EtOH	1 %	57	0.39 (022)	0.09 (005)	0.00 (000)	0.47 (027)	27	1.81	0.95	
NIC+RES	0.4560	45	0.69 (031) +	0.04 (002) -	0.07 (004) -	0.80 (036) +	36	1.86	1.65	0.65
NIC+RES	1.9000	42	0.76 (032) +	0.10 (004) -	0.12 (005) +	0.98 (041) +	41	2.02	2.00	1.05
NIC+RES	3.8000	52	0.62 (032) -	0.12 (006) -	0.04 (002) -	0.77 (040) +	40	1.77	1.60	0.60
NIC+RES	11.4000	37	0.38 (014) -	0.14 (005) -	0.05 (002) -	0.57 (021) -	20	2.30	1.10	0.15
HB*										
EtOH	1 %	60	0.33 (020) -	0.17 (010) -	0.00 (000) -	0.50 (030) -	28	2.00	0.95	
NIC+RES	0.4560	60	0.37 (022) -	0.10 (006) -	0.02 (001) -	0.48 (029) -	29	1.97	1.00	0.05
NIC+RES	1.9000	61	0.34 (021) -	0.10 (006) -	0.05 (003) -	0.49 (030) -	29	2.03	1.00	0.00
NIC+RES	3.8000	60	0.45 (027) -	0.22 (013) -	0.00 (000) -	0.67 (040) -	40	2.42	1.35	0.40
NIC treatments + RES [0.0110 mM]										
ST										
EtOH	1 %	60	0.45 (027) -	0.15 (009) -	0.05 (003) -	0.65 (039) -	38	2.34	1.30	
NIC+RES	0.0125	40	0.73 (029) +	0.00 (000) -	0.00 (000) -	0.73 (029) -	29	1.14	1.50	0.20
NIC+RES	0.0250	49	0.96 (047) +	0.27 (013) -	0.00 (000) -	1.22 (060) +	59	2.08	2.45	1.10
NIC+RES	0.0500	53	0.62 (033) -	0.04 (002) -	0.00 (000) -	0.66 (035) -	35	1.34	1.35	-1.10
HB										
EtOH	1 %	60	0.60 (036) -	0.08 (005) -	0.00 (000) -	0.68 (041) -	40	1.37	1.35	
NIC+RES	0.0125	67	0.54 (036) -	0.00 (000) -	0.04 (003) +	0.58 (039) -	39	1.54	1.20	-0.15

(*continued on next page*)

Table 6 (*continued*)

Compound Cross[b]		Number of flies	Spots per Fly (Number of Spots) Statistical Diagnosis[a]					Mean	Clone formation	
	Type		Small single spots	large single spots	Twin spots	Total spots	*mwh*clones	*mvh* clone size class	per 105 per cell	cells division[c]
			(1-2 cells)	(>2 cells)					observed	control connected
			m = 2	m = 5	m = 5	m = 5				
NIC+RES	0.0250	59	0.68 (040) -	0.07 (004) -	0.00 (000) -	0.75 (044) -	44	1.36	1.55	0.15
NIC+RES	0.0500	57	0.63 (036) -	0.05 (003) -	0.00 (000) -	0.68 (039) -	39	1.33	1.40	0.05

[a] Statistical diagnoses according to Frei and Würgler (1988) m: minimal risk multiplication factor for the assessment of negative results. For the final statistical diagnosis of all positive (+) and negative (-) results; the non-parametric Mann-Whitney and Wilcoxon *U*-test with significance levels α and β = 0.05 was used to exclude false positive or negative diagnoses [Frei and Würgler, 1995]. One side binomial test, significance levels α and β: significant results: + (α ≤ 0.05; no significant results: - (β ≤ 0.05).
[b] ST: standard cross; HB: high bioactivation cross.
[c] Clone frequencies per fly divided by the number of cells examined per fly (48,800) gives an estimate of formation frequencies per cell and per cell division in chronic exposure experiments [Frei and Würgler, 1995].*In HB cross the NIC [11.4 mM] + RES [0.0110 mM] treatment was lethal.

difference when these co-treatments were compared against each NIC or RES alone, which suggests an additive effect between NIC and RES in these co-treatments in the ST cross (Tables 4, 6, and 7).

*Protective effect of RES for NIC 0.*0125 mM *and 11.*4 mM *in the ST cross:* The 0.0125 mM and 11.4 mM NIC treatments were genotoxic, but we saw decreased damage for these NIC concentrations in co-treatment with 0.011 mM RES. The 0.0125 mM NIC + 0.011 mM RES co-treatment was genotoxic when compared against the dissolvent control. However, there was a significant decrease in small, large, and total spots frequencies when compared against the 0.0125 mM NIC treatment, which amounted to a 50% decrease in the damage caused by 0.0125 mM NIC (Tables 4, 6, and 7). The 11.4 mM NIC + 0.011 mM RES co-treatment was not genotoxic, while 11.4 mM NIC on its own was genotoxic when compared against the dissolvent control. This co-treatment had an increase in the frequency of large spots compared to the frequency of 0.011 mM RES, which suggests an increase in early damage that did not change the total spots frequency because the frequency of small spots decreased. However, there was a significant decrease in small and total spots frequencies when compared against the 11.4 mM NIC treatment (Tables 4, 6, and 7). These results suggest that 0.011 mM RES has a protective effect on the genotoxicity caused by 0.0125 mM and 11.4 mM NIC in the ST cross. In the HB cross, only the two highest NIC concentrations had an interaction with 0.011 mM RES.

*No interaction between RES and the 0.*0125 mM*, 0.*025 mM*, 0.*05 mM*, 0.*456 mM*, 1.*9 mM *NIC concentrations in the HB cross:* None of these co-treatments were genotoxic. Hence, there was no interaction between 0.011 mM RES and the 0.0125 mM, 0.025 mM, 0.05 mM, 0.456 mM, and the 1.9 mM NIC concentrations (Tables 4, 5, 6, and 7).

*Additive effect between NIC 3.*8 mM *and RES in the HB cross:* There was no genotoxic damage for the 0.011 mM RES + 3.8 mM NIC co-treatment compared to the dissolvent control in the HB cross. However, there was an increase in the frequency of large spots for this co-treatment when compared against the frequency of 3.8 mM NIC or 0.011 mM RES, which indicates early additive genotoxic damage and an interaction between both compounds (Tables 5, 6, and 7).

Increased toxicity for 11.4 NIC with RES in the HB cross: We obtained no data for the 0.011 mM RES + 11.4 mM NIC co-treatment in the HB cross because there were no survivors. These results show that in the HB cross 0.011 mM RES was not protective against the toxicity of 11.4 mM NIC and may have increased the toxicity of 11.4 mM NIC (Tables 5, 6, and 7). These results are different to what we found with the 0.0004 mM RES + 11.4 mM NIC co-treatment, in which 0.0004 mM RES was protective against the lethality of 11.4 mM NIC in the HB cross.

Involvement of the XM in NIC-RES interaction: Like what we saw with the 0.0004 mM RES co-treatments, the 0.011 mM RES concentration also showed a complex pattern of interactions with NIC that was not directly correlated with NIC concentration. We observed examples of increased damage, decreased damage and no effect, depending on the specific NIC-RES combination. We noticed more genotoxic damage in the 0.011 mM RES – NIC co-treatments in the ST cross than in the HB cross, which further suggests a role of the XM in the NIC-RES interaction.

Widespread mitotic alterations in the NIC-RES co-treatments in both crosses: In the ST cross, all co-treatment combinations, and comparisons except two showed significant differences in *mwh* clone size class distributions. Only 0.05 mM NIC + 0.011 mM RES compared to the dissolvent control and 0.025 mM NIC + 0.011 mM RES compared to 0.011 mM RES showed no effects on this distribution. In the HB cross, the *mwh* clone size class distribution was altered in a similar but not identical manner as in the co-treatments with the low NIC concentrations (Table 2). However, there were fewer NIC-RES combinations with altered *mwh* clone size class distribution for the co-treatments with the high NIC concentrations tested, most notably for the 0.011 mM RES co-treatments with either 0.456 mM or 1.9 mM NIC (Table 2). The 0.011 mM RES + 11.4 mM NIC co-treatment was lethal (Table 2).

3.4.3. Co-treatments results summary

Our hypothesis was that at 0.0004 mM RES would ameliorate any damaging effects of NIC, while RES 0.011 mM would increase NIC's damage. We found a protective effect for 0.011 mM RES in the ST cross in combination with the 0.0125 mM and 11.4 mM NIC concentrations, which were genotoxic on their own. However, RES in combination with some NIC concentrations that were not genotoxic on their own became genotoxic in combination, which suggested synergy or an additive interaction between NIC and RES. This happened with both RES concentrations tested. The most striking increase in damage happened with the 1.9 mM NIC + RES co-treatment with either 0.0004 mM RES or with 0.011 mM RES. We also found examples of no interaction between NIC and RES (Tables 1, 2, 3, 4, 5, and 6). Overall, the co-treatment results showed increased genotoxic effects of RES at the higher concentration, more damage in the ST cross than in the HB cross, a complex interaction between NIC and RES at different concentrations, and widespread alterations in cell division in both crosses and for most conditioned tested (Tables 1, 2, 3, 4, 5, 6, and 7).

4. Discussion

Tobacco addiction is a worldwide public health problem of top priority [4] in which NIC is the recognized addictive agent [2,3]. Several studies have shown that NIC induces oxidative stress [17,18]. While there are multiple accounts that oxidative stress is associated with

Table 7. Summary of results obtained in the standard (ST) and high bioactivation (HB) crosses of the *Drosophila* wing SMART after scoring marker-heterozygous flies (*mwh* +/+ *flr*[3], wild-type wings) treated with nicotine (NIC) at [0.0, 0.0125, 0.0250, 0.050, 0.456, 1.9, 3.8, 11.4 mM]; resveratrol (RES) at [0.0, 0.0004, 0.011 mM]; Milli-Q water and ethanol 1% as dissolvent controls.

Compound Cross[b]		Number of flies	Spots per Fly (Number of Spots) Statistical Diagnosis[a]					Mean	Clone formation	
	Type		Small single spots (1-2 cells) m = 2	large single spots (>2 cells) m = 5	Twin spots m = 5	Total spots m = 5	*mwh*clones	*mvh* clone size class	per 105 per cell observed	Cells division[c] control connected
RES [0.0004 mM]										
Co-treatments NIC [mM]/RES [0.0004 mM] vs. RES [0.0004 mM]										
RES	0.0004 mM	58	0.53 (031)	0.07 (004)	0.00 (0)	0.60 (035)	33	1.64	1.15	
NIC	0.456 + RES	33	0.61 (020) -	0.06 (002) -	0.00 (0) -	0.67 (022) -	22	1.45	1.35	0.2
NIC	1.900 + RES	22	0.77 (017) -	0.27 (006) +	0.00 (0) -	1.05 (023) +	23	1.87	2.15	1.0
NIC	3.800 + RES	38	0.42 (016) -	0.05 (002) -	0.00 (0) -	0.47 (018) -	16	1.06	0.85	-0.3
NIC	11.400 + RES	13	0.92 (012) -	0.38 (005) +	0.00 (0) -	1.31 (017) +	17	2.47	2.70	1.5
Co-treatments NIC [mM]/RES [0.0004 mM] vs. NIC										
NIC	0.456 mM	38	0.68 (026)	0.05 (002)	0.00 (0)	0.74 (028)	28	1.54	1.50	
NIC	0.456 + RES	33	0.61 (020) -	0.06 (002) -	0.00 (0) -	0.67 (022) -	22	1.45	1.35	-0.15
NIC	1.900	46	0.57 (026)	0.04 (002)	0.02 (001)	0.63 (029)	28	1.57	1.25	
NIC	1.900 + RES	22	0.77 (017) -	0.27 (006) +	0.00 (0) -	1.05 (023) +	23	1.87	2.25	1.0
NIC	3.800	32	0.62 (020)	0.03 (001)	0.03 (001)	0.69 (022)	22	1.45	1.40	
NIC	3.800 + RES	38	0.42 (016) -	0.05 (002) -	0.00 (0) -	0.47 (018) -	16	1.06	0.85	-0.55
NIC	11.400	22	1.27 (028)	0.23 (005)	0.05 (001)	1.55 (034)	34	1.71	3.15	
NIC	11.400 + RES	13	0.92 (012) -	0.38 (005) +	0.00 (0) -	1.31 (017) +	17	2.47	2.65	-0.5
RES [0.011 mM]										
Co-treatments NIC [mM]/RES [0.011 mM] vs. RES [0.011 mM]										
RES	0.011 mM	58	0.47 (027)	0.03 (002)	0.07 (004)	0.57 (033)	32	1.84	1.15	
NIC	0.456 + RES	45	0.69 (031) -	0.04 (002) -	0.07 (003) -	0.80 (036) -	36	1.86	1.65	0.5
NIC	1.900 + RES	42	0.76 (032) +	0.10 (004) -	0.12 (005) -	0.98 (041) +	41	2.02	2.00	0.85
NIC	3.800 + RES	52	0.62 (032) -	0.12 (006) -	0.04 (002) -	0.77 (040) -	40	1.77	1.60	0.45
NIC	11.400 + RES	37	0.38 (014) -	0.14 (005) +	0.05 (002) -	0.57 (021) -	20	2.30	1.15	0.00
o-treatments NIC [mM]/RES [0.011 mM] vs. NIC										
NIC	0.456 mM	38	0.68 (026)	0.05 (002)	0.00 (0)	0.74 (028)	28	1.54	1.5	
NIC	0.456 + RES	45	0.69 (031) -	0.04 (002) -	0.07 (003) -	0.80 (036) -	36	1.86	1.65	0.15
NIC	1.900	46	0.57 (026)	0.04 (002)	0.02 (001)	0.63 (029)	28	1.57	1.25	
NIC	1.900 + RES	42	0.76 (032) -	0.10 (004) -	0.12 (005) +	0.98 (041) +	41	2.02	2.00	0.75
NIC	3.800	32	0.62 (020)	0.03 (001)	0.03 (001)	0.69 (022)	28	1.45	1.40	
NIC	3.800 + RES	52	0.62 (032) -	0.12 (006) -	0.04 (002) -	0.77 (040) -	40	1.77	1.55	0.15
NIC	11.400	22	1.27 (028)	0.23 (005)	0.05 (001)	1.55 (034)	34	1.71	3.15	
NIC	11.400 + RES	37	0.38 (014) ↓	0.14 (005) -	0.05 (002) -	0.57 (021) ↓	20	2.30	1.10	-2.05
Co-treatments NIC [mM]/RES [0.011 mM] vs. RES [0.011 mM]										
RES	0.011 mM	99	0.58 (057)	0.05 (005)	0.04 (004)	0.67 (066)	65	1.58	1.35	
NIC	0.0125 + RES	40	0.73 (029) -	0.00 (000) -	0.00 (000) -	0.73 (029) -	29	1.14	1.50	0.15
NIC	0.0250 + RES	49	0.96 (047) +	0.27 (013) -	0.00 (000) -	1.22 (060) +	59	2.08	2.45	1.10
NIC		53	0.62 (033) -	0.04 (002) -		0.66 (035) -	35	1.34	1.35	0.00

(*continued on next page*)

Table 7 (*continued*)

Compound Cross[b]	Type	Number of flies	Spots per Fly (Number of Spots) Statistical Diagnosis[a]					Mean	Clone formation	
			Small single spots (1-2 cells) m = 2	large single spots (>2 cells) m = 5	Twin spots m = 5	Total spots m = 5	*mwh*clones	*mvh* clone size class	per 105 per cell observed	Cells division[c] control connected
	0.0500 + RES				0.00 (000) -					
Co-treatments NIC [mM]/RES [0.011 mM] vs. NIC										
NIC	0.0125	44	1.16 (051)	0.43 (019)	0.00 (000)	1.59 (070)	70	2.26	3.25	
NIC	0.0125 + RES	40	0.73 (029) ↓	0.00 (000) ↓	0.00 (000) -	0.73 (029) ↓	29	1.14	1.50	-1.8
	0.0250	41	0.66 (027)	0.00 (000)	0.02 (001)	0.68 (028)	28	1.21	1.40	
NIC	0.0250 + RES	49	0.96 (047) -	0.27 (013) -	0.00 (000) -	1.22 (060) +	59	2.08	2.45	1.05
NIC	0.050	21	0.67 (014)	0.10 (002)	0.05 (001)	0.81 (017)	17	1.47	1.66	
NIC	0.050 + RES	53	0.62 (033) -	0.04 (002) -	0.00 (000) -	0.66 (035) -	35	1.34	1.35	-0.31
HB										
RES 0.0004 mM										
RES	0.0004	87	0.52 (045)	0.06 (005)	0.01 (001)	0.59 (051)	51	1.84	1.20	
NIC	0.456 + RES	64	0.36 (023) -	0.11 (007) -	0.00 (000) -	0.47 (030) -	29	2.21	0.95	-0.25
NIC	1.900 + RES	49	0.29 (014) ↓	0.08 (004) -	0.02 (001) -	0.39 (019) -	18	2.33	0.75	-0.45
NIC	3.800 + RES	78	0.59 (046) -	0.05 (004) -	0.03 (002) -	0.67 (052) -	52	1.63	1.35	0.15
NIC	11.400 + RES	10	0.40 (004) -	0.00 (000) -	0.00 (000) -	0.40 (004) -	04	1.25	0.80	-0.40
NIC	0.456	99	0.38 (038)	0.07 (007)	0.03 (003)	0.48(048)	47	1.64	0.95	
NIC	0.456 + RES	64	0.36 (023) -	0.11 (007) -	0.00 (000) -	0.47 (030) -	29	2.21	0.90	-0.05
NIC	1.900	58	0.47 (027)	0.05 (003)	0.07 (004)	0.59 (034)	34	1.82	1.20	
NIC	1.900 + RES	49	0.29 (014) -	0.08 (004) -	0.02 (001) -	0.39 (019) -	18	2.33	0.75	-0.45
NIC	3.800	69	0.36 (025)	0.06 (004)	0.00 (000)	0.42 (029)	28	1.61	0.85	
NIC	3.800 + RES	78	0.59 (046) +	0.05 (004) -	0.03 (002) -	0.67 (052) +	52	1.63	1.40	0.55
RES 0.011 mM										
RES	0.011	59	0.36 (021)	0.02 (001)	0.02 (001)	0.39 (023)	23	1.43	0.80	
NIC	0.456 + RES	60	0.37 (022) -	0.10 (006) -	0.02 (001) -	0.48 (029) -	29	1.97	1.00	0.20
NIC	1.900 + RES	60	0.34 (021) -	0.10 (006) -	0.05 (003) -	0.49 (030) -	29	2.03	1.00	0.20
NIC	3.800 + RES	60	0.45 (027) -	0.22 (013) +	0.00 (000) -	0.67 (040) -	40	2.42	1.35	0.55
Co-treatments NIC [mM]/RES [0.011 mM] vs. NIC										
NIC	0.456	60	0.33 (020)	0.08 (005)	0.02 (001)	0.43 (026)	25	1.64	0.85	
NIC	0.456 + RES	60	0.37 (022) -	0.10 (006) -	0.02 (001) -	0.48 (029) -	29	1.97	1.00	0.15
NIC	1.900	58	0.47 (027)	0.05 (003)	0.07 (004)	0.59 (034)	34	1.82	1.2	
NIC	1.900 + RES	60	0.34 (021) -	0.10 (006) -	0.05 (003) -	0.49 (030) -	29	2.03	1.0	0.20
NIC	1.900 + RES	60	0.34 (021) -	0.10 (006) -	0.05 (003) -	0.49 (030) -	29	2.03	1.0	0.20
NIC	1.900 + RES	60	0.34 (021) -	0.10 (006) -	0.05 (003) -	0.49 (030) -	29	2.03	1.0	0.20
NIC	3.800 + RES	60	0.45 (027) -	0.22 (013) +	0.00 (000) -	0.67 (040) +	40	2.42	1.35	0.55
Co-treatments NIC [mM]/RES [0.011 mM] vs. RES [0.011 mM]										
RES	0.011 mM	60	0.73 (044)	0.07 (004)	0.02 (001)	0.82 (049)	48	1.56	1.65	
NIC	0.0125 + RES	67	0.54 (036) -	0.00 (000) -	0.04 (003)-	0.58 (039) -	39	1.54	1.20	-0.45

(continued on next page)

Table 7 (*continued*)

Compound Cross[b]		Number of flies	Spots per Fly (Number of Spots) Statistical Diagnosis[a]					Mean	Clone formation	
	Type		Small single spots	large single spots	Twin spots	Total spots	*mwh*clones	*mvh* clone size class	per 105 per cell	Cells division[c]
			(1-2 cells)	(>2 cells)					observed	control connected
			m = 2	m = 5	m = 5	m = 5				
NIC	0.0250 + RES	59	0.68 (040) -	0.07 (004) -	0.00 (000) -	0.75 (044) -	44	1.36	1.55	-0.10
NIC	0.0500 + RES	57	0.63 (036) -	0.05 (003) -	0.00 (000) -	0.68 (039) -	39	1.33	1.40	-0.25
Co-treatments NIC [mM]/RES [0.011 mM] vs. NIC										
NIC	0.0125	60	0.68 (041)	0.05 (003)	0.02 (001)	0.75 (045)	42	1.14	1.45	
NIC	0.0125 + RES	67	0.54 (036) -	0.00 (000) -	0.04 (003)-	0.58 (039) -	39	1.54	1.2	-0.25
NIC	0.0250	60	0.65 (039)	0.03 (002)	0.00 (000)	0.68 (041) -	41	1.29	1.4	
NIC	0.0250 + RES	59	0.68 (040) -	0.07 (004) -	0.00 (000) -	0.75 (044) -	44	1.36	1.55	0.15
NIC	0.050	60	0.77 (046)	0.08 (005)	0.00 (000)	0.85 (051) -	51	1.61	1.75	
NIC	0.050 + RES	57	0.63 (036) -	0.05 (003) -	0.00 (000) -	0.68 (039) -	39	1.33	1.4	-0.35

[a] Statistical diagnoses according to Frei and Würgler (1988) m: minimal risk multiplication factor for the assessment of negative results. For the final statistical diagnosis of all positive (+) and negative (-) results; (↓) significant decrease; the non-parametric Mann-Whitney and Wilcoxon U-test with significance levels α and β = 0.05 was used to exclude false positive or negative diagnoses [Frei and Würgler, 1995]. One side binomial test, significance levels α and β: significative results: + (α ≤ 0.05; no significant results: - (β ≤ 0.05).

[b] ST: standard cross; HB: high bioactivation cross.

[c] Clone frequencies per fly divided by the number of cells examined per fly (48,800) gives an estimate of formation frequencies per cell and per cell division in chronic exposure experiments [Frei and Würgler, 1995].

genotoxicity [19,22], most studies have examined the genotoxic effects of NIC *in vitro* [23,27]. RES has been attributed multiple beneficial properties such as being an antioxidant, a free radical scavenger and increasing longevity [32,33,41,74]. However, very few studies have examined if RES could modulate the effects of NIC [49–51]. Moreover, the effect of RES on the putative genotoxic effect of NIC has not been studied *in vivo*. Hence, we aimed to determine NIC's genotoxic potential using the SMART wing spot test in *Drosophila melanogaster* and whether RES could inhibit (RES 0.0004 mM) or increase (RES 0.011 mM) the genotoxicity of NIC.

Our *D. melanogaster* SMART wing spot test results showed that, as expected, NIC was genotoxic in the ST cross, but only at specific concentrations. NIC caused lethality at the highest concentration in the HB cross and was cytotoxic in both crosses, which shows that the XM was involved in modulating these effects. On the other hand, RES was cytotoxic in both crosses at the 0.0004 and 0.011 mM concentrations, and weakly genotoxic in the ST cross. When NIC and RES were tested in combination we saw an increase in genotoxicity with both RES concentrations tested and a complex interaction between NIC and RES at different concentrations. The combination of NIC and RES resulted in alterations of the cell division at most combinations tested. The XM modulated the effects described. A discussion by treatment follows.

4.1. Nicotine

Several studies *in vitro* have shown that NIC can induce genotoxic damage [23,24,26,27,68], alter the cell cycle [20], and decrease cell viability [21]. Here we tested NIC's genotoxicity *in vivo*. We hypothesized that NIC would be genotoxic and would alter the cell division, as expected from *in vitro* study results. Our data showed a non-linear relationship in the genotoxic effects of NIC in the ST cross, with the lowest (0.0125 mM) and the highest (11.4 mM) concentrations causing genotoxic damage, while the intermediate concentrations (0.025 mM, 0.5 mM, 0.456 mM, 1.9 mM, 3.8 mM) were not genotoxic. In addition, the 11.4 mM NIC concentration was semi lethal in both crosses but had higher lethality in the HB cross. This may be explained because of NIC's chemical structure, which generates free radicals and ROS [10,75]. NIC has been shown to generate ROS and induce oxidative stress [76,77]. However, no NIC concentration was genotoxic in the HB cross. This could be explained because it is well established that the HB cross is better able to withstand oxidative stress damage [56,78].

The ST cross has basal and inducible activity of the Cyp450 enzymes [78]. One possible explanation for these results is that genotoxic damage occurred because the lowest NIC concentration was too low to recruit the XM or engage DNA repair mechanisms sufficiently. The intermediate concentrations were high enough to recruit the XM and DNA repair mechanisms and the NIC was cleared fast enough that any damage NIC may have caused was likely countered, resulting in no genotoxic damage detected on the SMART test. However, at the highest NIC concentration, in this cross, the XM and DNA repair mechanisms may have been insufficient to clear and counteract all the damage induced by NIC, and genotoxic damage was detected. These observations fit a biphasic curve of stress containment, which is consistent with a response in which either insufficient stress (a low NIC-dose) or excessive stress (a high NIC dose) yield a DNA damage response (genotoxicity), while the doses in between yield a no damage.

Results from the HB cross, in which the *Cyp450s* are over-expressed [62,78] showed no genotoxic damage in the surviving flies, at any of the concentrations tested. This supports the idea that increasing the XM and clearance of NIC [79,80,81] was sufficient to counteract the genotoxic damage NIC could have induced.

We infer that NIC induced genotoxicity or aneuploidy at a low concentration because several studies of the effects of NIC *in vitro* have shown that NIC induces damage at low concentrations. It has been demonstrated that 1 and 10 μM NIC causes micronuclei and DNA damage by inducing oxidative stress [67]. But also, NIC has been shown to be genotoxic in *in vitro* assays at concentrations larger than 0.5 μM in human lymphocytes [27]. In addition, NIC at 1–100 μM concentration has been shown to produce chromosomal aberrations and sister chromatid exchange [24]. Sobkowiak and Lesicki (2009) found a dose-response effect on the DNA fragmentation induced by NIC at 1, 10, and 100 μM concentrations on the comet assay in *C. elegans* [82]. They found that the 100 μM concentration was genotoxic.

We also found that the cell division was significantly altered. There was more significant alteration in the ST cross than in the HB cross. If this was due to oxidative damage, this would explain why there was less damage in the HB cross, which has elevated levels of expression of XM phase I and II genes and antioxidant enzymes [56,78,83].

4.2. Resveratrol

RES has been shown to have antioxidant properties, radical scavenging activity, and protective activity [32–34,40]. In *Drosophila melanogaster*, RES was protective against certain chemicals that induce oxidative stress [43,45,46]. However, RES was not protective against other oxidant compounds [47,48]. In particular, 0.011 mM RES had been previously found to be genotoxic and increase the genotoxic damage of 4-NQO in *Drosophila melanogaster* [48]. Because of the discrepancy in the literature results regarding RES [42–48], in this study we aimed to re-examine whether RES at the 0.011 mM concentration was genotoxic. We also wanted to test a second concentration that has not been reported to be genotoxic and choose 0.0004 mM RES. As expected, the 0.0004 mM RES concentration was not genotoxic. However, the 0.011 mM concentration was not conclusively genotoxic in this investigation. This result was surprising because the 0.011 mM RES concentration has been reported to be genotoxic in various assays including in the ST cross on the SMART wing spot test [48], the micronuclei assay, chromosomal aberrations test and the hybridization of sister chromatids assay [84,85,86]. This discrepancy may be explained by differences in the genotoxicity of the 1% EtOH dissolvent control used. We noticed that the number of spots detected in the dissolvent control was closely correlated to that of RES.

Both RES concentrations tested altered the *mwh* clone size class distribution, from which we inferred that there were mitotic alterations [54]. Studies about the beneficial effects of RES at similar concentrations *in vitro* and *in vivo* could provide an explanation for these results. It has been shown that RES 0.01 mM can reduce tumors on mouse skin [87,88]. RES is also known to modulate apoptosis by activating certain caspases [65,89,90] or activating p53, a tumor suppressor protein that plays an important role in stopping the cell cycle [91] and trigger DNA repair [92]. Chen *et al.* (2013) reported that RES at 0.001 and 0.005 mM promoted cell proliferation and viability, while at 0.02 mM inhibited cell survival of *A549* human adenocarcinoma cells [89]. RES could also be altering the cell division through a process of auto-oxidation, which generates O_2, H_2O_2, quinones and semi-quinones that can be cytotoxic [93,94].

4.3. NIC and RES co-treatments

A few studies have tested the interaction between NIC and RES and showed that RES can decrease NIC's effects, both *in vitro* and *in vivo* [49–53]. Our results demonstrate that RES modulates the effects of NIC. However, these RES concentrations had different effects on the toxicity and genotoxicity of the NIC concentrations tested. We detected unexpected effects in the ST cross and with the higher RES concentration (0.011 mM). For example, in the 0.0125 mM NIC + 0.011 mM RES and 11.4 mM NIC + 0.011 mM RES co-treatments in the ST cross, there was a clear decrease in the frequency of small and total spots, which can be interpreted as a protective effect of RES 0.011 mM on the genotoxicity induced by NIC at the 0.0125 mM or 11.4 mM concentrations. Saliently, the genotoxic damage caused by 0.0125 mM NIC diminished by 50% in combination with 0.011 mM RES. This may be explained by: a) the antioxidant effect of RES [95]; b) an increase in apoptotic damage [65,87,88]; c) RES activation of sirtuins, which could have diminished NIC's cellular damage [96]. However, in the 11.4 mM NIC + 0.011 mM RES co-treatment compared against RES there was an increase in the frequency of large spots (Table 5) which could indicate an interaction between NIC and RES that led to an increase in the early genotoxic damage. All these effects correlate with our data that the cell division was affected in all the previously mentioned conditions tested.

On the other hand, the 0.025 mM NIC + 0.011 mM RES, 0.456 mM NIC + 0.011 mM RES, 1.9 mM NIC + 0.0004 mM RES, 1.9 mM NIC + 0.011 mM, 3.8 mM NIC + 0.011 mM RES and 11.4 mM NIC + 0.0004 mM RES were genotoxic in the ST cross, while only the 0.456 mM NIC (0.456) + 0.0004 RES and 3.8 mM NIC + 0.0004 RES co-treatments were genotoxic in the HB cross. These results could indicate a pro-oxidant effect by RES when in combination with NIC and influenced by the Cyp450s levels. RES could have antioxidant and cytotoxic effects, as a function of its concentration, the length of exposure, and the compound with which it becomes conjugated [97].

4.4. Relevance to humans

The sequence similarity between *D. melanogaster* and mammals at the nucleotide and amino acid sequence levels is about 40% between homologs and as high as 80–90% for conserved functional domains [98]. The high correspondence between the proteins and genes involved in the xenobiotic metabolism in *D. melanogaster* and humans further support that *D. melanogaster* is an adequate model organism for research to identify the biological processes involved in the metabolism of drugs and toxins [99]. Moreover, more than 60% of human disease-causing genes have an ortholog in *Drosophila melanogaster* [100]. This high conservation at the genetic and molecular levels has allowed research to evaluate the effects of drugs of potential clinical relevance to humans that may be toxic, affect survival, or be involved in cancer, obesity, sleep and neurological disorders, polycystic disease and traumatic brain injury [101]. Similarly, *Drosophila* has been successfully used to explore novel therapeutic strategies [102]. The *Drosophila* model and the SMART assay in particular have been successfully used to detect carcinogenic chemicals, with reports in the literature including research on tamoxifen [103]; https://monographs.iarc.who.int/agents-classified-by-the-iarc/], benzidine [104] and bleomycin [105], among many others. In addition, van Schaik y Graf [106,107] used the SMART assay in *Drosophila* to show six tricyclic antidepressants (TCAs) were genotoxic. A subsequent epidemiological study by Sharpe *et al.* [108] showed an increase in breast cancer rate ratios associated with heavy TCA exposure 11–15 years later. A recent review paper of this model further supports its applicability to as a human disease model for genotoxic testing [109].

5. Conclusions

Most of the genotoxic effects we observed were on small spots, which suggests late damage, cell arrest or aneuploidogenic genotoxic damage [54]. If such late damage was due to the XM, then the genotoxicity of the NIC 0.0125 mM concentration in the ST cross could be explained by having basal Cyp450 levels, while in the HB cross, NIC 0.0125 mM was metabolized more quickly, and hence it was not genotoxic. Our results show complex relationships between NIC and RES and provide evidence that the interaction between oxidant and putative antioxidant compounds cannot be assumed as protective as a general event. Based on the results of this investigation we conclude that the genotoxic effects of RES are weak, which explains the results from Dueñas *et al.* [48], who reported RES as a genotoxic agent, while we found in this work that RES was not genotoxic. This happens when the differences in spot frequencies between RES and its dissolvent control (EtOH) are small or limited. In our study, NIC was genotoxic at concentrations in which the XM could be unable to effectively cope with NIC's toxicity. These results suggest a modulation between NIC and RES that depends on their concentrations and on the XM. This modulation is not always beneficial and can lead to increased genotoxic and toxic damage. In addition, both RES and NIC alone and in combination altered the cell division, which shows they can cause damage independently of their genotoxic effects. These complex interactions show that more research is needed *in vivo* to uncover possible unanticipated harmful effects of RES.

Declarations

Author contribution statement

Velázquez-Ulloa N.A & Heres-Pulido M.E: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dueñas-García I.E.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Santos-Cruz L.F.: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Browning A; Ferderer G; Garfias M & Perry H.H: Performed the experiments.

Carmona-Alvarado C; Estrada-Guzmán J.C & Gómez-Loza B: Performed the experiments; Analyzed and interpreted the data.

Castañeda-Partida L.: Contributed reagents, materials, analysis tools or data.

Magaña-Acosta M.J. & Durán-Díaz A: Analyzed and interpreted the data.

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The authors declare no conflict of interest.

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