Chapter

The *w*/*w*$^+$ Somatic Mutation and Recombination Test (SMART) of *Drosophila melanogaster* for Detecting Antigenotoxic Activity

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Abstract

Genotoxicological studies are emerging as fundamental for knowing the hazards to our genome, to our health. *Drosophila melanogaster* is one of the preferable organisms for toxicological research considering its metabolic similarities (viz. on dietary input, xenobiotic metabolizing system, antioxidant enzymes and DNA repair systems) to mammals. Accordingly, somatic mutation and recombination tests (SMARTs) of *D. melanogaster* are fast and low-cost in vivo assays that have shown solid results evaluating genotoxicity. The *w*/*w*$^+$ SMART uses the *white* (*w*) gene as a recessive marker to monitor the presence of mutant ommatidia (eye units), indicating the occurrence of point mutations, deletions, mitotic recombination or/and nondisjunction. Additionally, several studies used SMARTs to assess antigenotoxicity, with some using the *w*/*w*$^+$ SMART. We reviewed the state of the art of the *w*/*w*$^+$ SMART used for antigenotoxicity analysis, focusing on published results, aiming to contribute to the conception of a reliable protocol in antigenotoxicity. As such, genotoxic agents with known action mechanisms, as streptonigrin (oxidative stress inducer), were used as a genotoxic insult for proving the antigenotoxic effects of natural substances (e.g. seaweeds), demonstrating the presence of antimutagens in their composition. These antigenotoxicity studies are crucial for promoting preventive measures against environmental genotoxics that affect humans daily.

Keywords: genotoxicity test, *w*/*w*$^+$ SMART, eye-spot test, *Drosophila melanogaster*, streptonigrin, genotoxic agent, oxidative stress, DNA damage, ROS inhibition, antigenotoxicity, antimutagens, dietary antioxidants

1. Introduction

The environmental emergency is largely related to environmental toxicology. Each day, new molecules are synthesized, or natural molecules are intensively produced that enter in ecosystems and affect them at all levels. Nowadays there are circulating in living organisms thousands of substances that did not exist 100 years ago, with somewhat unpredictable consequences. As such, more than 159 million chemical substances are registered in the Chemical Abstracts Service (CAS), with

approximately 4000 new substances being registered daily [1]. As a controlling measure, the EU Commission created, in 2004, a network (NORMAN network) of laboratories, research centres and organizations for monitoring the emerging environmental substances [1].

Environmental toxicology encompasses exposure to toxic substances whether through the air we breathe, the food we eat, the water we drink and the clothes we wear or through the skin, cosmetics, etc. There is also radiation exposure, which also has harmful effects, and is much more problematic today than some years ago. The planet is poisoned, affecting the air, the water, the soil and the food we produce, which causes serious problems to human health and ecosystems. It is hoped that worldwide awareness of this reality will be achieved, and the focus of humanity's greatest concerns will be on the cleansing of the planet by eliminating or at least greatly reducing the produced toxic agents.

This whole problem greatly affects DNA, causing DNA damage (genotoxicity), affecting DNA repair mechanisms and causing mutations when damage is not properly repaired. In the short term, this genome instability leads to diseases such as cancer, degenerative diseases, fertility decrease and other problems. In the long term, we may see the emergence of new diseases due to new mutations in the germ line, which, if recessive, may take several generations until there is a chance of homozygosis, where rare diseases may arise. All combined may affect the life expectancy of several species, causing an environmental collapse. Preventive strategies are indispensable to reduce the heavy burden on national healthcare systems and families. The most effective is a healthy lifestyle including diet, as an antigenotoxic diet reduces DNA damage and all the associated diseases. Antigenotoxic activities include inactivation of genotoxic compounds, by several mechanisms and increasing repair capacity, decreasing the effectiveness of a genotoxic. While DNA damage is clearly implicated as the initiating event in most cancers, the link is not a simple one. Most damage is removed by repair enzymes before it can interfere with the process of DNA replication and introduce mutations. Given a carcinogenic exposure, the individual variation in the capacity for DNA repair is therefore likely to be an important factor in determining cancer risk.

Over the years, many investigations in DNA damage and DNA repair mechanisms were made, in vitro and in vivo, aiming to know our environment and thus identifying the harmful compounds to our genome, to our health, leading to preventive actions such as prohibiting the commercialization of certain drugs, construction materials, foods and drinks. Genotoxicological studies using cell cultures and animals are essential for increasing human's wellbeing, since they display solid results in showing the genotoxicity of compounds and should be standardized (with optimal test conditions) for increasing their reproducibility and precision.

2. *Drosophila melanogaster* in toxicological research

Drosophila melanogaster is currently being used as one of the preferable organisms for toxicological research [2]. According to current knowledge, the use of *D. melanogaster* as a model organism respects the principles of animal welfare (3Rs), since ethical matters do not urge when using this organism [2, 3]. Considering the metabolic pathways responsible for dietary input (including nutrient uptake, digestion, absorption, storage and metabolism) [4], the xenobiotic metabolizing system, the antioxidant enzymes and the DNA repair systems of *D. melanogaster*, which are analogous to those of mammals, *D. melanogaster* emerges as an optimal replacer of higher animals in toxicological studies [2, 5]. Furthermore, contrasting

with in vitro methods, *D. melanogaster* has the advantage of enabling a more solid extrapolation at the organism level [3].

D. melanogaster exposure to toxic agents leads to the alteration of simple life traits, which are perturbed negatively, such as development time, number of eclosed individuals, sex ratio, adult body size, fertility and others [6, 7]. These life traits can be assessed as a way of measuring the toxicological effects of a given drug, food, drink and so on. However, as science progresses and hazards are targeted in a more specific way, genotoxicological studies with *D. melanogaster* were developed, aiming to identify environmental hazards inducing damages to genome, *i.e.* genotoxic agents. In this way, genotoxicological studies with *D. melanogaster* deal with the assessment of changes in genetic material through various assays, such as germ line mutation assays, somatic mutation assays, the chromosomal aberration assay, the micronucleus test, the comet assay and DNA sequence-based assays, among others. In particular, somatic mutation and recombination tests (SMARTs) have proven to be a good tool for detecting a broad range of genetic alterations quickly and inexpensively [2, 8].

2.1 Somatic mutation and recombination tests of *D. melanogaster*

The somatic mutation and recombination tests of *D. melanogaster* have shown excellent results in assessing the genotoxicity of several and diversified compounds in somatic cells. Originally, in the 1980s, the SMART could be performed by four different assays, but only two of them made it through to the present day: the wing-spot test and the eye-spot test (or *w*/*w*$^+$ SMART) [9]. The wing-spot test was firstly described by Graf and Würgler [10] and the *w*/*w*$^+$ SMART by Würgler and Vogel [11], with both showing high values of sensitivity, specificity and accuracy.

Briefly, in the late embryogenesis, larval structures are set, and groups of diploid cells of undifferentiated epithelium, imaginal discs, are formed in the embryo [12]. Then, upon the ending of the larval stages, pupa emerges, and metamorphosis takes place upon systemic hormonal regulation, with the histolysis of the larval organs and differentiation of the imaginal discs into adult structures [13, 14]. Accordingly, the exposure of imaginal discs to genotoxic agents may lead to genetic alterations (the product of DNA damage) capable of being transmitted to daughter cells upon mitosis. These genetic alterations can be phenotypically manifested in the adults in structures such as the wings and the eyes, which can be assessed according to the wing-spot test and the eye-spot test, respectively. The loss of heterozygosity (LOH) for specific genetic markers in heterozygous individuals allows the quantification of DNA damage/level of genotoxicity in the adult tissues by visual scoring [9, 15].

Between the two types of SMART currently used, from the practical point of view, the *w*/*w*$^+$ SMART can be assayed with six different strains, as firstly shown by Vogel and Nivard [16], contrasting with only two strains available for the wing-spot one; in the *w*/*w*$^+$ SMART, a standardized genotoxic agent, inducing a high genotoxicity without toxic effects, streptonigrin (further focused on the chapter) [17], is available and has proved its effectiveness. Nevertheless, since the wing-spot test allows the visual scoring of wings over time, considering that wings are mounted/preserved on slides, opposite from what happens in the *w*/*w*$^+$ SMART, where eyes have to be analysed quickly since no preserving actions are available (time limited scoring), a greater number of studies have been performed using the wing-spot test (**Table 1**). Henceforward, as a measure of further exploring the potential of this test and increasing its number of studies, the *w*/*w*$^+$ SMART will be focused.

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
Abraham [18]	Wing-spot	Cyclophosphamide (CPH) Diethylnitrosamine (DEN) Mitomycin C (MMC) Procarbazine (PRO) Urethane (URE)	Coffee	+ + + − +
Alaraby et al. [19]	Wing-spot	Potassium dichromate (PD)	CeO_2 NPs Cerium sulphate	+ +
Alaraby et al. [20]	Wing-spot	Potassium dichromate (PD) Ethyl methanesulfonate (EMS) Potassium dichromate (PD) Ethyl methanesulfonate (EMS)	CuO NPs Copper oxide	+ +
Amkiss et al. [21]	Eye-spot	Methyl methanesulfonate (MMS)	Fennel plant fruit extracts	+
Anter et al. [22]	Wing-spot	Hydrogen peroxide	Virgin Olive oil Triolein Tyrosol Squalene	+ + + +
Anter et al. [23]	Wing-spot	Hydrogen peroxide	Red table grapes	+
Anter et al. [24]	Wing-spot	Hydrogen peroxide	Phenols: apigenin, bisabolol, protocatechuic acid	+ + +
Aydemir et al. [25]	Wing-spot	Fotemustine	Amifostine	+
Cápiro et al. [26]	Eye-spot	Methyl methanesulfonate (MMS) Ethylnitrosourea (ENU) Juglone (JG) Dimethylbenz(a)anthracene (DMBA)	*Cymbopogon citratus*	+ + + +
Demir and Marcos [27]	Wing-spot	Potassium dichromate	Boron nitride nanotubes	+
De Rezende et al. [28]	Wing-spot	Doxorubicin (DXR)	Grape seed proanthocyanidins	+
De Rezende et al. [29]	Wing-spot	Doxorubicin (DXR)	Dibenzylbutyrolactolic lignan(−)-cubebin	+/−
Drosopoulou et al. [30]	Wing-spot	Mitomycin C (MMC)	Chios mastic products: verbenone α-terpineol linalool trans-pinocarveol	+ + + −
El Hamss et al. [31]	Wing-spot	Urethane (URE)	Turmeric	+
Fernandes et al. [32]	Wing-spot	Doxorubicin (DXR) Benzo(a)pyrene (B(a)P)	Vitexin	+ +
Fernandez-Bedmar and Alonso-Moraga [33]	Wing-spot	Hydrogen peroxide	Green sweet pepper Red sweet pepper Green hot pepper Red hot pepper Capsaicin Capsanthin Lutein	+ + − + + + +

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
Fernández-Bedmar et al. [34]	Wing-spot	Hydrogen peroxide	Citrus juices Hesperidin Limonene	+ + +
Fernandez-Bedmar et al. [35]	Wing-spot	Hydrogen peroxide	Tomato Lycopene	+ +
Fernández-Bedmar et al. [36]	Wing-spot	Hydrogen peroxide	Garlic Onion Diallyl disulphide Dipropyl disulphide	+ + + +
Ferreira et al. [3]	Eye-spot	Streptonigrin (SN)	*Grateloupia turuturu* *Porphyra umbilicalis*	+ +
Graf et al. [37]	Wing-spot	Urethane (URE) Methyl urea + sodium nitrite	Instant coffee Ascorbic acid Catechin	+ + +
Guterres et al. [38]	Wing-spot	Doxorubicin (DXR)	*Momordica charantia*: aerial parts Fruit	– +
Idaomar et al. [39]	Wing-spot	Urethane (URE)	Essential oils from: *Helichrysum italicum*, *Ledum groenlandicum*, *Ravensara aromatica*	+ + +
Kylyc and Yesilada [40]	Wing-spot	Mitomycin C (MMC)	Dried mycelia from: *Trametes versicolor* *Pleurotus ostreatus*	+ +
Laohavechvanich et al. [41]	Wing-spot	Urethane (URE)	Bird pepper Red chili spur pepper Green bell pepper Green sweet pepper	+ + + +
Lozano-Baena et al. [42]	Wing-spot	Hydrogen peroxide	*Brassica carinata* Sinigrin	+ +
Marques et al. [43]	Eye-spot	Streptonigrin (SN)	*Ulva rigida* *Fucus vesiculosus* *Gracilaria* species	+ + +
Martinez-Valdivieso et al. [44]	Wing-spot	Hydrogen peroxide	Lutein β-Carotene Zeaxanthin Dehydroascorbic acid Yellow zucchini Light green zucchini	+ + + + + +
Mateo-Fernandez et al. [45]	Wing-spot	Hydrogen peroxide	Caramel color class IV	+
Merinas-Amo et al. [46]	Wing-spot	Hydrogen peroxide	Choline	+
Mezzoug et al. [47]	Wing-spot	Urethane (URE)	*Origanum compactum* essential oil	+
Niikawa et al. [48]	Wing-spot	Mitomycin C (MMC)	Salicylic acid Salicyluric acid Gentisic acid Gentisuric acid 2,3-Dihydroxybenzoic acid	– + + + +

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
Niikawa et al. [49]	Wing-spot	Mitomycin C (MMC)	Salicylic acid	–
			Salicyluric acid	+
			Gentisic acid	+
			Gentisuric acid	+
			2,3-Dihydroxybenzoic acid	+
Oliveira et al. [50]	Wing-spot	Doxorubicin (DXR)	Metformin	+
Orsolin et al. [51]	Wing-spot	Doxorubicin (DXR)	Simvastatin	+
Pádua et al. [52]	Wing-spot	Mitomycin C (MMC)	*Terminalia actinophylla* extracts	+
		Ethyl methanesulfonate (EMS)		+
Patenkovic et al. [53]	Wing-spot	Methyl methanesulfonate (MMS)	Sage tea	+
Patenkovic et al. [54]	Wing-spot	Methyl methanesulfonate (MMS)	Gentian tea	–
Prakash et al. [55]	Wing-spot	Ethyl methanesulfonate (EMS)	Caffeine	+
Prakash et al. [56]	Wing-spot	Methyl methanesulfonate (MMS)	*Dioscorea pentaphylla*	+
Rizki et al. [57]	Wing-spot	Potassium dichromate (PD)	Sodium selenite	+
Romero-Jiménez et al. [58]	Wing-spot	Hydrogen peroxide	*Matricaria chamomilla*	+
			Tilia cordata	+
			Mentha piperita	+
			Mentha pulegium	+
			Uncaria tomentosa	+
			Valeriana officinalis	+
Sarıkaya et al. [59]	Wing-spot	Ethyl methanesulfonate (EMS)	Boron	+
Savić et al. [60]	Wing-spot	Methyl methanesulfonate (MMS)	Royal Sun Agaricus extract	–
Sukprasansap et al. [61]	Wing-spot	Urethane (URE)	Eggplants	+
Taira et al. [62]	Wing-spot	2-AAF	*Agrocybe cylindracea*	+
		Aflatoxin B1	*Lentinula edodes*	+
		DMBA	*Pleurotus ostreatus*	+
		IQ		+
		MeIQx		+
		MNU		+
		NDMA		+
		4NQO		+
		2-AAF		-
		Aflatoxin B1		+
		DMBA		–
		IQ		–
		MeIQx		–
		MNU		+
		NDMA		+
		4NQO		+
		2-AAF		+
		Aflatoxin B1		+
		DMBA		+
		IQ		+
		MeIQx		+
		MNU		+

Reference	SMART type	Genotoxic agent	Substance tested as antigenotoxic	Response
		NDMA 4NQO		+ +
Tasset-Cuevas et al. [63]	Wing-spot	Hydrogen peroxide	Borage seed oil Gamma linolenic acid	+ +
Toyoshima et al. [64]	Wing-spot	Sun and UV light	Sunscreens: SPF 20 SPF 40 SPF 60	+ + +
Valadares et al. [65]	Wing-spot	Doxorubicin (DXR)	Propolis (water extracts)	+
Valente et al. [66]	Eye-spot	Streptonigrin (SN)	Thalassotherapy products	+

The type of test, wing- or eye-spot, the used genotoxic agents, as well as the information about the antigenotoxic potential of the tested substances (response: + antigenotoxic activity; − no antigenotoxic activity or synergistic genotoxic activity) is presented.

Table 1.
Published studies focusing the antigenotoxic evaluation of several types of chemicals, nanoparticles and plants/seaweeds/seeds/oils using somatic mutation and recombination tests (SMARTs).

3. *w*/*w*$^+$ SMART (eye-spot test)

D. melanogaster presents two symmetrically positioned eyes in its head. Each eye consists of repeated hexagonal arrays of approximately 750–800 ommatidia (eye units formed upon differentiation of imaginal discs), homogenous in size and regularly spaced, with each ommatidium being constituted by 14 cells (8 photoreceptor cells, 4 cone cells and 2 primary pigment cells) [67]. Between each two ommatidia, six secondary pigment cells, three tertiary pigment cells and three mechanosensory bristle complexes are present [67]. The adult eye of *D. melanogaster* is particularly used in toxicological assays since subtle defects in ommatidia development are amplified, by mitosis, several hundred times in the eye [68]. Therefore, it is quite simple to detect genetic alterations changing its pigmentation.

The basis of the *w*/*w*$^+$ SMART is the *white* (*w*) gene located at the position 1.5 of the X chromosome. This gene is used as a recessive genetic marker to monitor the presence of mutant ommatidia/spots, indicating the occurrence of LOH by deletions, point mutations, mitotic/somatic recombination (the most frequent) or/and nondisjunction (chromosome losses) in somatic cells (**Figures 1** and **2**) [9, 16]. These genetic events are known to display a significant role in the induction of carcinogenesis [69]. Accordingly, when wild-type females (*w*$^+$/*w*$^+$; red eyes) are crossed with white-eyed males (*w*/Y; eyes without pigmentation), or vice versa (*w*/*w* with *w*$^+$/Y), a heterozygous offspring is developed for females (*w*$^+$/*w*; red eyes). However, if the offspring is exposed to genotoxic agents in its development phase, the presence of white/mutant phenotype spots in the red eyes may occur (**Figures 1** and **2**). In addition, when crossing wild-type females with white-eyed males, males' eyes can also be analysed, although somatic recombination should not be considered in this case [9]. The difference between females and males scoring will provide quantitative information on somatic recombination [9].

Moreover, Vogel and Nivard [69, 70] designed a more refined, as well as time-consuming, version of the *w*/*w*$^+$ SMART, which allows the detection of chromosomal aberrations in late larval stages. However, and according to Marcos and Sierra [9], the ratio of results obtained/time consumption is low in comparison with the

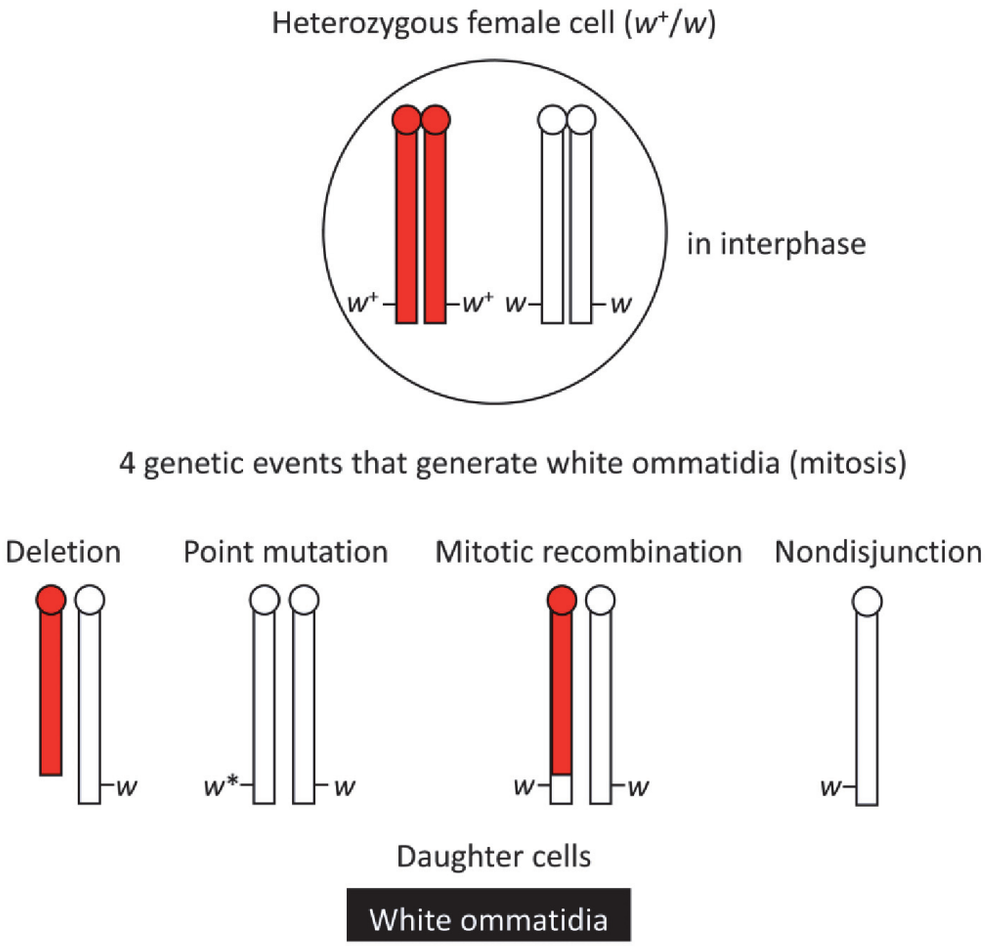

Figure 1.
Scheme of the possible four types of genetic alterations that generate white ommatidia in a heterozygous D. melanogaster *female for the white (w) gene. In the scheme, the heterozygous female cell has two X chromosomes with two chromatids each (duplicated DNA in interphase) and daughter cells have two X chromosomes but only one chromatid each (except for nondisjunction). The X chromosomes in red carry the w^+ allele (dominant) and those in white carry the w allele (recessive), however there are a few exceptions that will be described below. The position of the alleles in the X chromosomes is represented in a purely illustrative, non-exact way. w^* is a mutated wild-type expressing white phenotype. In the development phase of a heterozygous female for the w gene (w^+/w), genetic alterations may be induced in the imaginal discs and, upon cell division, daughter cells with mutant/white phenotype ommatidia may appear. The genetic alterations that cause mutant phenotypes are: deletion in one X chromosome including the white locus (in the wild-type allele); point mutation in the wild-type allele by substitution, insertion, or deletion; mitotic recombination between chromatids of the homologous X chromosomes, that replaces the wild-type locus by a mutant locus; nondisjunction, that causes the loss of the chromosome with the wild-type allele.*

original version of the assay, making it less efficient in the laboratorial routine. Thus, the original version of the assay continues to be the main choice when performing w/w^+ SMART.

3.1 Antigenotoxicity with w/w^+ SMART

w/w^+ SMART was, in its original concept, used for the genotoxicological evaluation of several chemical agents, directed to unveiling the action mechanisms behind their genotoxic activities [17, 71–73]. As such, alkylating agents, such as methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS) and ethylnitrosourea (ENU), are between the chemicals that induce a great number of mutant ommatidia in *D. melanogaster* [72]. Even so, and considering the study from Gaivão and Sierra [17], a quinone-based antibiotic, streptonigrin (SN), showed its potential to induce a great level of genotoxicity (increased number of mutant

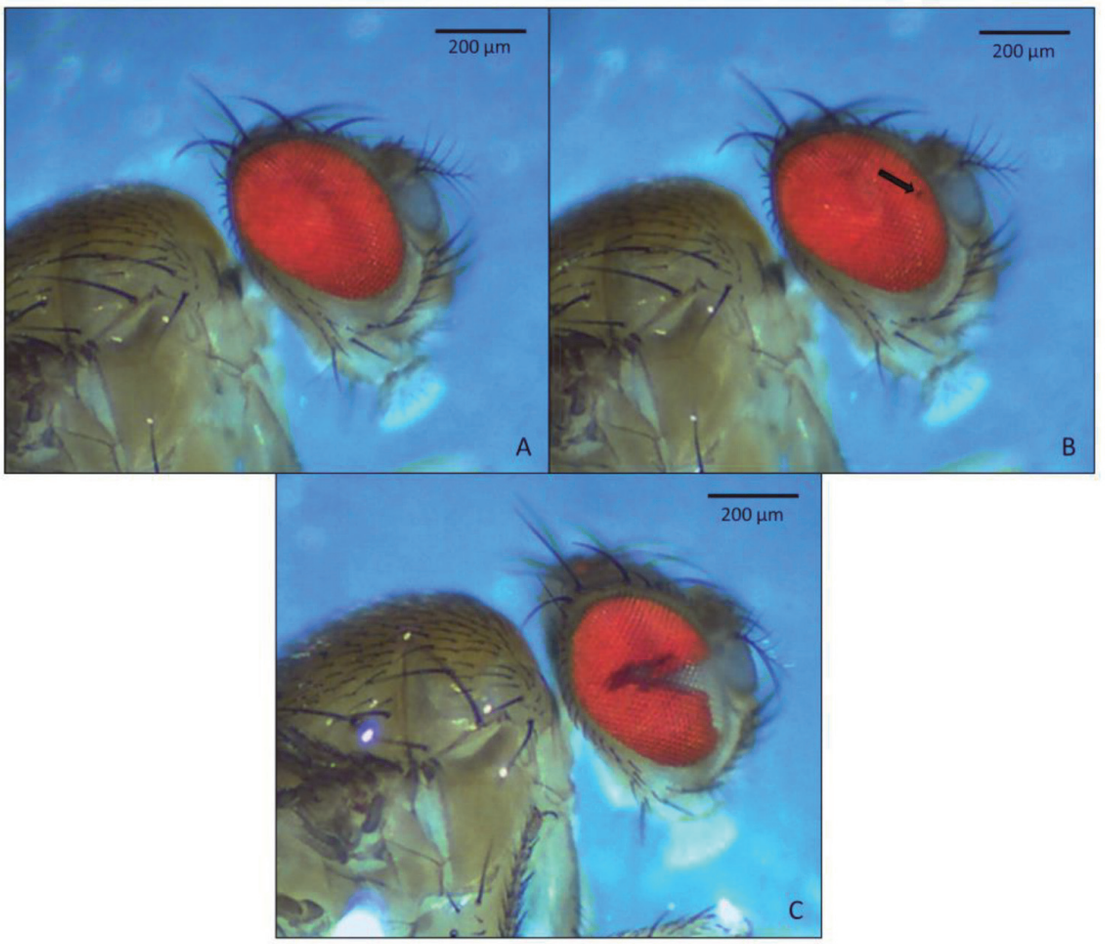

Figure 2.
Wild-type eyes of D. melanogaster *(females) at the stereoscopic microscope (80× magnification). (A) An eye without mutant spots, (B) an eye with a dark spot affecting one to two ommatidium(a) (marked by a black arrow) and (C) an eye with a spot affecting innumerable ommatidia. White mutant spots appear as black when surrounded by pigmented/red ommatidia.*

ommatidia) without toxic effects (at 20 μM) in the *w*/*w*⁺ SMART, making it a suitable genotoxic insult for this assay. SN, in the presence of certain metal cations (Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Cd^{2+} and/or Au^{2+}), binds to DNA establishing SN-metal-DNA complexes, known as DNA adducts [74–76] (**Figure 3**). Upon the binding, the quinone reduces, via one or two e^- (NADH as a cofactor), producing a semiquinone or a hydroquinone, respectively. Semiquinone reacting with O_2 leads to the production of O_2^- and quinone regeneration. Hydroquinone can lead to the production of H_2O_2, while quinone is regenerated (**Figure 3**). In consequence, OH can be produced by the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow OH + OH^- + Fe^{3+}$) and by the Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow OH + OH^- + O_2$), leading to oxidative stress [74–76]. The production of reactive oxygen species (ROS), and the prolonged SN linkage to DNA, can lead to the inhibition of DNA (and RNA) synthesis, induce unscheduled DNA synthesis, promote DNA strand breaks as well as inhibit topoisomerase II [77]. Chromosomal aberrations may occur upon mutagenic events, creating genomic instability that can culminate into carcinogenic events [76] (**Figure 3**).

Among the processes related to genotoxicity, with an increased relevance in the last years, the analysis of antigenotoxicity is probably the most important one. The search for antigenotoxic agents that could prevent or counteract the harmful consequences of the exposure to DNA damaging agents has increased exponentially lately [78–80]. Since most of the possible antigenotoxic agents are components of natural products that could be included in the diet, the analysis of their properties

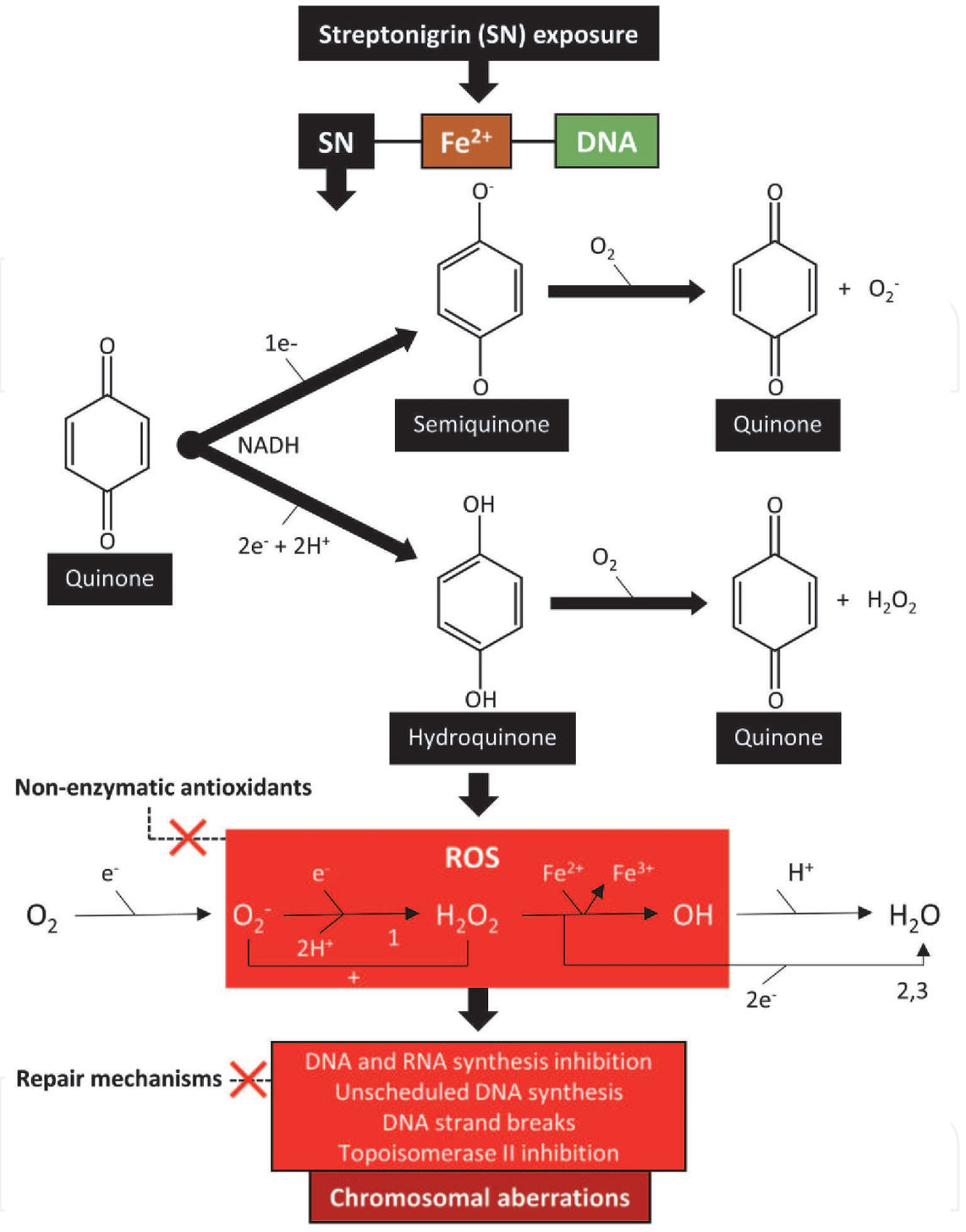

Figure 3.
Simplistic scheme of the genotoxic activity of streptonigrin (SN) on an animal cell. Cell exposure to SN leads to the formation of DNA adducts [SN + metal cation (such as Fe^{2+}) + DNA]. SN's quinone groups are reduced (NADH as a cofactor) to semiquinone and hydroquinone that, in the presence of O_2, lead to the formation of O_2^- and H_2O_2, respectively, both with quinone regeneration (vicious cycle). Thus, by chemical reactions (such as the Fenton and Haber-Weiss ones), OH is produced, the most severe reactive oxidative species (ROS). In this case, the antioxidants (endogenous enzymatic and non-enzymatic, and dietary inputs) are not capable of avoiding excessive ROS formation and progression, as well as communicating to repair mechanisms for repairing the induced genetic damages that may lead to chromosomal aberrations. (1) Superoxide dismutase (SOD); (2) catalase (CAT); (3) glutathione peroxidases.

should be performed in in vivo experiments. As so, *Drosophila* fulfils all the requirements for this analysis, specifically when using SMARTs. In fact, there are numerous published studies using *D. melanogaster* in antigenotoxicity analyses, and most of them are using SMARTs, especially with the wing-spot test (**Table 1**).

Focusing on the *w*/*w*$^+$ SMART performed for antigenotoxicity testing, there are a few studies evaluating the antigenotoxic potential of lemongrass extracts [26]; fennel plant fruit extracts [21]; red, green and brown seaweeds [3, 43]; and thalassotherapy products (containing seaweeds) [66].

Ferreira and Marques [3] and Marques and Ferreira [43] studied the exposure of *D. melanogaster* [*Oregon-K* (*OK*) strain] to a chronic treatment (from egg to adult eclosion) with media (Formula 4-24® Instant Drosophila Medium) supplemented with red, green or brown seaweeds and SN (at 20 μM). Reductions in the number of mutant ommatidia were shown in individuals cotreated with seaweed and SN in relation to the positive control. Thus, protective properties of seaweeds were exerted against the genotoxic insult of SN, demonstrating antigenotoxic potential. Even more, some species displayed antigenotoxic effects against the spontaneous genotoxicity (without SN insult) of *D. melanogaster*. The authors also refer the possible phytochemicals acting as antimutagens that include vitamins, phenolic compounds, pigments and polysaccharides. These phytochemicals, which may promote their action in a synergetic way, may inhibit ROS triggered by SN activity, acting as dietary antioxidants [3] (**Figure 3**). Their mechanisms of action may include ROS scavenging, donation of electrons and/or protons to endogenous enzymatic and/or non-enzymatic antioxidants for converting ROS to H_2O and/or chelating metal ions responsible for producing OH (Fenton reaction inhibition) [34, 81]. In line, using the same conditions, Valente and Borges [66] showed the antigenotoxicity of thalassotherapy products (with seaweeds) against SN. Once more, the potential of seaweeds as dietary antioxidants/antimutagens, as well as the potential of SN as an optimal inducer of chromosomal aberrations quantifiable by the SMART, was demonstrated. Longevity-promoting properties were also displayed upon seaweed supplementation which, according to free radical and mitochondrial theories of ageing, may be a collateral effect of the dietary antioxidants that modulate the enzymatic antioxidants and exert direct antioxidant-scavenging actions [3, 66].

MMS (at 1 mM) was used as a genotoxic insult against a fennel plant fruit aqueous extract [21]. The positive control showed a great number of induced mutant ommatidia, proving the results from Vogel and Nivard [72], and the fennel extract showed antigenotoxic activity against MMS. According to the authors, and considering the mutagenic activity of MMS, an alkylating agent, consisting of direct interactions with DNA bases that induce mutagenic events, fennel may possess antimutagens that interact directly with the methyl radical groups of MMS and inactivate them in such a manner that they cannot bind to DNA as effectively to induce their mutagenic activity. The antimutagenic properties displayed by fennel may be related to components of its essential oil [21]. In a similar way, Cápiro and Sánchez-Lamar [26] demonstrated the antigenotoxic potential of lemongrass decoction extracts against different genotoxics, MMS, ENU, juglone (JG) and dimethylbenz(a)anthracene (DMBA), that exhibit different mechanisms of action. According to the authors, the lemongrass extract modulated the genotoxic action of the alkylating agents MMS and ENU by interacting with them directly or/and with their mutagenic derivatives. Regarding JG, a naphthoquinone that induces ROS production in an analogous way to SN, damages were reduced upon exposure to the decoction extract by probably inhibiting ROS production, by sequestrating/inhibiting ROS activity or/and activating intracellular defence mechanisms. For DMBA, as it needs metabolic activation by microsomal enzymes, the extract may have interfered with the microsomal enzymatic system for avoiding DMBA activation. Overall, lemongrass extract acted as an antimutagen in the protection of DNA.

In fact, SMART can be assayed using different test conditions, including the *D. melanogaster* strain (OK strain has potential for genotoxicity testing; presents high

susceptibility to ROS, mainly due to a low activity of antioxidant enzymes, being more sensitive to increase its antioxidant status upon intake of dietary antioxidants [3, 73]), treatment method (chronic or acute and pre-, co- and post-treatments), genotoxic agent (should always be chosen among those with a known mechanism of action; an example is SN) and sample size. For more details on the methodological approaches of SMARTs, see the protocol from Marcos and Sierra [9].

4. Conclusions

In vitro and especially in vivo genotoxicity testing of substances such as foods, drinks, drugs and herbicides is fundamental for increasing humans' knowledge on the hazards that we may be exposed to. In this way, upon the identification of a substance/compound as genotoxic, priorities should be focused on avoiding this genotoxic or, at least, when the exposure is unavoidable, preventing our metabolism from damages to DNA that can culminate in mutagenic events and, in a later stage, on carcinogenesis. Upon in vitro testing, in vivo genotoxicological assays, such as w/w^+ SMART in *D. melanogaster*, are great tools for evaluating the antigenotoxic potential of a given substance/compound, considering optimal test conditions. The ultimate objective of these tests is to promote the dietary intake of antimutagens, since they are essential for reinforcing our metabolic defences towards genotoxic events, especially the ones that may be produced by strong exogenous agents. Foods, teas, nutraceuticals and others who are richly composed of dietary antimutagens should be of daily intake, considering that there is an increasing threat of new chemical substances with genotoxic potential every day.

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Conflict of interest

The authors declare no conflict of interest.

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