

Figure 4 shows the corrected frequencies of total spots from the two types of progeny. The corrected spot frequencies in experimental series were obtained by subtracting the number of spontaneous spots; hence, the corrected frequencies correspond to an estimate of the mutant spots actually induced by the compound. Using both two markers to identify the progeny phenotype, data show that the corrected frequency of total spots in inversion-free flies was clearly higher than the corrected frequency in inversion-carrier flies.

In the present study, the observed genotoxic effect suggests that CO, in addition to aneuploidogenic activity, is a mutagen that induces spots in the SMART of *Drosophila*. The use of a second marker (ebony) improved the classification of the phenotypes of the progeny analyzed.

More experimental evidence needs to be obtained to explore whether the nicks on the wings induced in treated flies could be associated to aneuploidogenic activity and, in consequence, whether the alteration in the border of wings could be an auxiliary tool to identify compounds with aneuploidogenic activity.

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**Mammals replacement: *Drosophila* is a reliable option for the screening of anti-inflammatory activity.**

**Muñoz-Moya, Armando, Patricia Ramos-Morales\*, and Adriana Muñoz-Hernández.**

Laboratorio de Genética y Toxicología Ambiental, Facultad de Ciencias, Universidad Nacional Autónoma de México. Av. Universidad 3000, México D.F. CP 04510, MX.;

\*Correspondence, Tel/Fax (55)-56-22-51-94; e-mail: [prm@hp.fciencias.unam.mx](mailto:prm@hp.fciencias.unam.mx).

## Introduction

Inflammation induced by chemical, physical, or biological agents implies both vascular and cellular reaction mediated by chemical factors. Chronic inflammation has been associated with several steps preceding cancer as cellular transformation, cellular proliferation, tissue invasion, angiogenesis, and metastasis (Mantovani, 2005). The cancer's risk increases in patients showing inflammatory processes (Ohshima *et al.*, 2003), and in cancerous patients the inflammation accelerates tumor growth and cancer progression.

Methods for screening compounds for anti-inflammatory activity use rodent models, which previously were injected with croton oil or similar agents as swollen inductors. After a period of

time, rodents are given a second injection with the potential anti-inflammatory compound. To determine the anti-inflammatory activity, a compound should produce around a 60% reduction in the weight of ear's discs (mice) or the paw oedema mean (rats) (Falodun *et al.*, 2006; Usman *et al.*, 2008).

Regulatory guidelines concerning the use of animals for biological research focus on ensuring that the 3Rs, Reduction, Refinement and Replacement, are properly applied. All the users of animals for basic or routine research are committed to explore alternative methodologies to reach research aims, but reducing or avoiding the use of mammals (ESF, 2001).

In this report we propose the use of *Drosophila melanogaster* and the Somatic Mutation and Recombination Test (SMART) as a tool for screening compounds for anti-inflammatory activity. *Drosophila*'s biology is well known, sharing more than 60% of its genes with humans. In addition to individual genes, complexes and metabolic pathways are conserved in flies and humans (Mackay and Anholt, 2006).

In *Drosophila*, precursor cells of the wings are genetically determined after embryonic development, proliferate by mitosis during larval stage, and reach their differentiation until metamorphosis, when each one of the cells on the wing blade produces a hair or trichome. Using recessive markers that modify the trichome phenotype, it is possible to determine whether in some of the precursor cells the loss of heterozygosity happened giving rise to a mutant spot in the adult wing. The size of the spot depends on the number of cell cycles occurring after the loss of heterozygosity in the original cell. Events such as point mutation, deletion, non-disjunction, and mitotic recombination can lead to a mutant spot formation (Graf *et al.*, 1984). Even though the SMART is a methodology originally designed for genotoxicity screening, we hypothesized it could be an option for the screening of anti-inflammatory activity, too. In spite of the kind of mutagen that organisms could be exposed to, the genotoxic effect produced depends on: the capacity of the mutagen to alter the genetic material, but also on the oxidative damage associated to the induction of detoxification mechanisms. For years, it's been known that the Reactive Oxygen Species (ROS) have an active role in the mutagenicity and DNA damage induction (Xu *et al.*, 1999).

The tumor promoter 12-*O*-tetradecanoil phorbol-13-acetate (TPA) produces inflammatory effects in the skin of rodents, being used in a routine way to induce paw oedema in rats or ear's inflammation in mice for the screening of anti-inflammatory compounds (Park *et al.*, 2008). Inflammation response implies oedema and hyperplasia, but also the induction of pro-inflammatory enzymes and cytokines; increasing the expression and activity of Ornithine Decarboxylase (ODC), Cyclooxygenase-2 (COX-2), Phospholipase-2 (PLA<sub>2</sub>), Protein Kinase C (PKC), Nitric Oxide Synthase (iNOS), and others. During this process, reactive oxygen species (ROS) are liberated (Murakami *et al.*, 2000; Seo *et al.*, 2002). In the 1980's, several reports showed that DNA damage as chromosomal aberrations can be induced by free radicals reactions and ROS indirectly produced by TPA (Emerit and Cerutti, 1982; Emerit and Lahound-Maghani, 1989).

On the other hand, glucocorticoids (GCC) have been effective in reducing the inflammation reaction in a wide spectrum of diseases, including allergic diseases as asthma, arthritis, and autoimmune disturbances, among others. Frequently GCC are the more effective therapies accessible, but their use is restricted due collateral negative effects (Barnes, 1995). In the cells, GCC activate the glucocorticoids receptors (GRs), GRs regulate directly the transcription of around 10 to 100 particular genes (Gronemeyer, 1992; Beato, *et al.*, 1995); however, many more genes could be indirectly regulated through the interaction with other transcription factors. GR activated forms a homodimer, which joins to DNA on consensus sequences named glucocorticoids response elements (GRE), which located in the 5' side upstream of promoter region of genes responding to GCC. The GR-GRE association modifies the rate transcription and results in the induction or repression of genes. Since numerous genes without GRE can be restrained by GCC, it has been suggested that

other mechanisms could be involved (Barnes, 1998). GR can interfere with the protein synthesis reducing the mRNA stability through increasing the specific ribonuclease transcription, which nicked mRNA in the region rich in AU sequences in the 3' side untranslated and hence shortening the lifetime of mRNA. In summary, GCC can control the inflammation by inhibiting numerous steps of the inflammatory process, *i.e.* increasing the transcription of anti-inflammatory genes (Lipocortin-1,  $\beta_2$ -adrenoreceptor, receptor antagonist IL-1, and IL-1R2) or decreasing the transcription of inflammatory genes (Cytokines, Quimiokinas, iNOS, COX-2, PLA<sub>2</sub>, Endothelin-1, and adhesion molecules, among others) (Barnes, 1996; Barnes and Adcock, 1993).

In this paper we show that the SMART can be an alternative methodology for the screening of anti-inflammatory activity based on the genetic damage induced by oxyradicals associated with the inflammation reaction induced by TPA.

## Materials and Methods

### *Chemical compounds*

12-*O*- tetradecanoylphorbol-13-acetate (TPA) [CAS 16561-29-8], Cortisone (COR) [CAS 53-065], and Ethanol (EtOH) [64-17-5] were purchased from Sigma (Sigma-Aldrich Química, MX); Dimethyl sulfoxide (DMSO) [CAS 67-68-5] from Baker (Baker Mallinckrodt Baker, MX); and microcrystalline cellulose from Merck (Merck, Germany). All other compounds used were equivalent to analytic grade of Sigma.

### *Strains, crosses and culture medium used for the SMART of Drosophila*

*D. melanogaster* strains with genetic markers in the left arm of chromosome 3 were used, including 1) females  $flr^3/In(3LR)TM3, ri p^p sep bx^{34e} e^s Bd^s$ , abbreviated ( $flr^3/TM3, Bd^s$ ). The gene marker  $flr^3$  (3-38.8, map units m.u.) is lethal when homozygous at organismal level, but at somatic cell level it alters the trichome form showing it as a flame. The maintenance of  $flr^3$  gene requires the balancing chromosome TM3, which carries as marker the allele Beaded-Serrate  $Bd^s$  (3-91.9 m.u.), originally called Serrate (*Ser*), dominant and also lethal when homozygous. 2)  $mwh/mwh$  males. Multiple wing hair marker,  $mwh$  (3-0.0 m.u.), is recessive and in homozygotes allows the production of multiple trichomes (2 to 14 or more) compared with one trichome by cells on wild-type flies. For a detailed marker description refer to Lindsley and Zimm (1992). The flies were maintained at 25°C on flasks with standard medium (composed of 10g of agar-agar, 72.5g of baker yeast, 135g of sucrose, and 105.g of corn flour each 1000 ml water).

Parental cross was done with 72 hr old  $flr^3/TM3, Bd^s$  virgin females and 48 hr old  $mwh/mwh$  males.

### *Experimental procedure*

Egg collection was done by transferring the parental cross to flasks with fresh standard medium (enriched with a drop of fresh baker yeast) for 2 hr and withdrawing the parental flies afterwards. Three days later, larvae of  $72 \pm 1$  hr were recovered by floating in a 20% sucrose solution (Graf *et al.*, 1984; Nöthiger, 1970) and put in groups of 100-150 into glass tubes (2 cm diameter, 10 cm high) with a nylon cloth on one extreme. After this, every tube was introduced in a 10 ml glass container with 0.4 g of cellulose (powder) and 0.3 ml of the solution to be assayed for one hour. After the exposure time the larvae were gently washed with tap water at room temperature and transferred to flasks with fresh standard media to continue their development. In order to choose

the concentrations for the combined treatment, both TPA and COR were assayed separately. For TPA, larvae were exposed for 1 hr to the 0.275–0.55 mM TPA dissolved in 5% DMSO solution. In independent experiments, larvae were exposed for 1 hr to 0.56–5.6 mM COR dissolved in 5% Ethanol solution (EtOH). For the combined treatment, larvae were exposed for 1 hr to 5% DMSO or 0.55 mM TPA, gently washed with tap water, and exposed for 1 hr to 5% EtOH or 1.4, 2.8 mM COR, afterwards washed again and transferred to flask with fresh standard medium to continue their development. For each assay, each experiment was performed at least two times.

### *Wing analysis*

The flies recovered were sacrificed by anesthesia excess with diethyl ether and fixed in 70% EtOH. Wings from ten females and ten males were mounted by couples on glass slides using Fauré solution (Graf *et al.*, 1984). Ventral and dorsal wing surfaces were observed at 40× with an optical microscope (Nikon, YS100, Japan), and each one of the mutant spots was scored according to the wing section where it was located (García-Bellido and Merriam, 1971). The spots were grouped in three categories: 1) simple small spots (one or two cells affected), 2) simple large spots (three or more cells affected), and 3) twin spots (formed by the *flr*<sup>3</sup> and *mwh* markers). The final sample size scored was 200 wings.

### *Statistical analysis*

The frequency of spots from experimental and control series was compared according to the multiple decision procedure of Frei and Würzler (1988). A significant increase in the frequency of spots is obtained whether the frequency of spots in experimental series is  $m$  (multiplication factor) times the frequency of that from the control flies. For the small spots and the sum of all the spots  $m = 2$ , and for large and twin spots which happen with low frequency,  $m = 5$  (Frei and Würzler, 1995). To compare the dispersion on the distribution of the number of spots-per-fly the non-parametrical test of Kruskal-Wallis and the Multiple Comparison Dunn's Test were used. A change in the distribution is considered when significant differences were found at  $p < 0.05$ . Statistical analysis was performed on SPSS 11.0 software (SPSS Inc. Chicago, Illinois, USA).

## **Results**

### *Survival index*

To determine the toxicity after treatment, a survival index (IS) was obtained as the average of the number of flies recovered from experimental series compared with the total of flies in their concurrent control (which was considered 100%). Toxicity associated to treatments was rather moderate. Flies treated as larvae with 0.275 mM TPA showed an IS of 73%, while all other TPA treatments produced IS above 80%. On COR treatments the survival indices obtained were in the range of 83–93%. Then, COR was less toxic than TPA in the concentrations assayed. On the other hand combined treatments had a similar 98% survival index; which shows the TPA-COR interaction which decreases the toxicity induced by TPA (Table 1).

Table I. Survival Index (average  $\pm$  estandar error), Frequency of spots per wing in + *flr*<sup>3</sup>/*mwh* + flies treated as larvae with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), Cortisone (COR) and the combined treatment with TPA - COR..

[mM] Compound	Survival Index (SI)	Spots per wing				
		Number of Wings	Small Spots m=2 b	Large Spots m=5 b	Twin Spots m=5 b	Total Spots m=2 b
(72 X 1 hr) DMSO 5% TPA	1	200	59 (0.29)	5 (0.03)	1 (0.005)	65 (0.32)
0.275	0.73 $\pm$ 0.10	200	116 (0.58) +	10 (0.05) i	1 (0.005) -	127 (0.63) +
0.344	0.81 $\pm$ 0.09	200	113 (0.56) +	13 (0.06) +	1 (0.005) -	127 (0.63) +
0.413	0.82 $\pm$ 0.08	200	119 (0.60) +	8 (0.04) i	2 (0.01) -	129 (0.64) +
0.55	0.85 $\pm$ 0.15	200	136 (0.68) +	10 (0.05) i	2 (0.01) -	148 (0.74) +
(72 X 1 hr) EtOH 5 % COR	1	200	60 (0.30)	7 (0.04)	0 (0.000)	67 (0.34)
0.56	0.93 $\pm$ 0.07	200	54 (0.27) -	11 (0.05) -	2 (0.01) -	67 (0.34) -
1.4	0.84 $\pm$ 0.12	200	54 (0.27) -	5 (0.03) -	1 (0.005) -	60 (0.30) -
2.8	0.84 $\pm$ 0.11	200	53 (0.26) -	10 (0.05) -	1 (0.005) -	64 (0.32) -
5.6	0.83 $\pm$ 0.15	200	59 (0.29) -	8 (0.04) -	1 (0.005) -	68 (0.34) -
Combined (72 X 1 X 1 hr)						
TPA 0.55 - EtOH 5 %	1	200	136 (0.68) +	15 (0.08) +	0 (0.000) -	151 (0.75) +
TPA 0.55 - COR 1.4	0.98 $\pm$ 0.02	200	48 (0.24) -	6 (0.03) -	2 (0.01) -	56 (0.28) -
TPA 0.55 - COR 2.8	0.98 $\pm$ 0.02	200	45 (0.22) -	6 (0.03) -	0 (0.000) -	51 (0.25) -

b, Statistical diagnosis according to Frei y Wurgler (1988) Mutat. Res. 203:297-308; -, negative; +, positive; i, inconclusive; m =, multiplication factor; p = 0.05.

### Wing SMART

*D. melanogaster* SMART results are summarized in Table 1. TPA had genotoxic activity and increased significantly the frequency of small and total spots at all concentrations assayed (Figure 1a). The increase of large spots was only observed after a concentration of 0.344 mM TPA ( $p < 0.05$ ). The genotoxicity of this treatment modifies the distribution of the number of spots per fly. The spotless fly proportion was smaller on TPA-treated flies, and the number of spots-per-fly was significantly increased at all concentrations tested ( $p < 0.001$ ) (Figure 1b). Instead COR did not show genotoxic activity at any concentration assayed (Figure 2a) and, hence, the spots-per-fly number remained the same (Figure 2b).

On treatment TPA - COR the frequency of small and total spots was smaller when COR concentration was increased (1.4 and 2.8 mM), from 0.28 to 0.25, respectively. Comparing these results with the spot frequency on TPA control (0.75) indicates the amelioration of TPA genotoxic activity when COR is present (Table 1, Figure 3a). In addition the spot-per-fly number distribution was modified and more flies without spots were recovered ( $p < 0.001$ ) (Figure 3b).

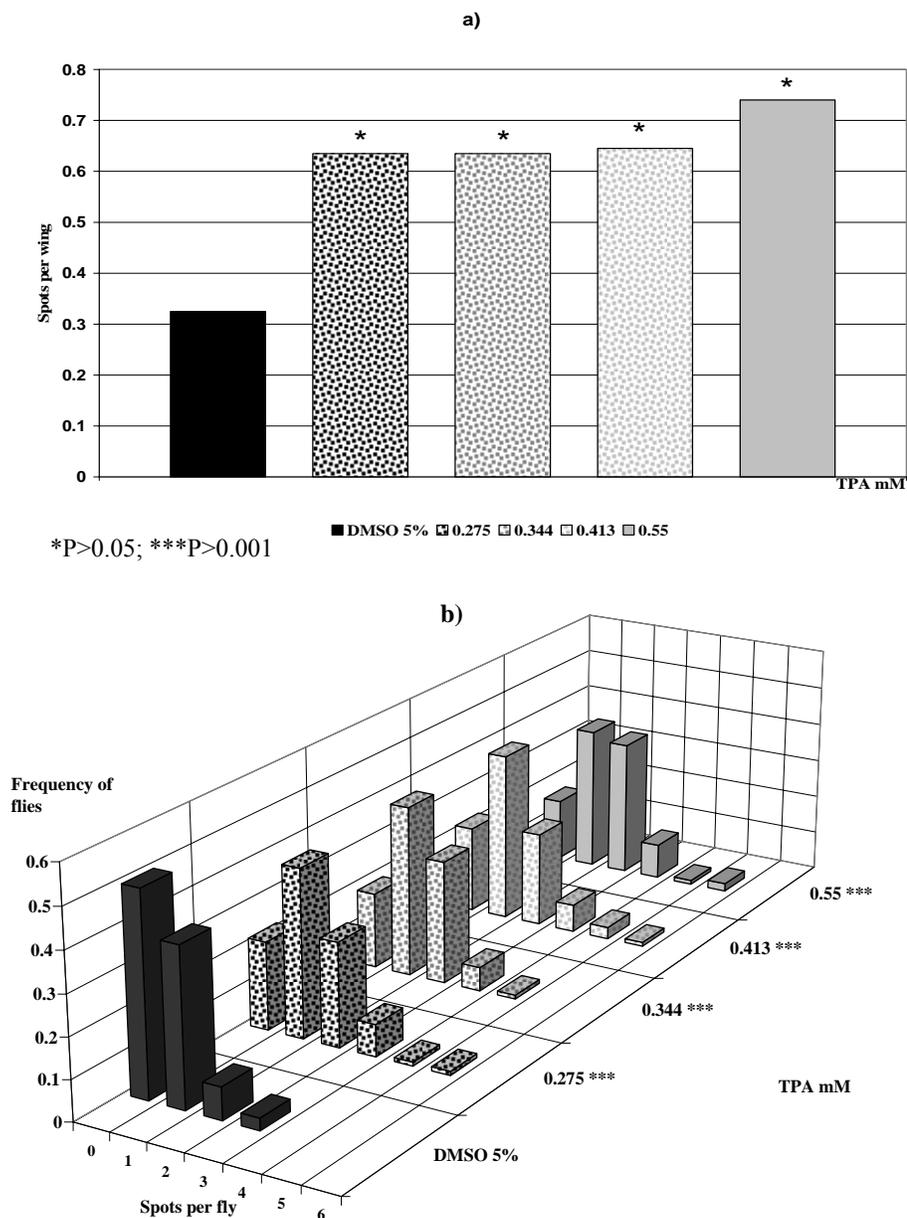


Figure 1. a) Frequency of total spots, and b) spots per fly distribution from flies exposed to TPA.

## Discussion

In this report, we try to show that *Drosophila melanogaster* can be used for the screening of compounds with anti-inflammatory activity, taking advantage that for years it has been accepted that factors producing ROS can indirectly induce genotoxic damage. Another side, GCC are effective reducers of inflammation in mammals.

In *Drosophila*, the ROS inducer TPA shows low toxicity for exposed larvae, but increases the frequency of spots in the wings at all the concentrations assayed ( $p < 0.05$ ). This effect was reported

previously by Emerit and Lahound-Maghani (1989) and is consistent with the induction of SCE in V79-4 Chinese hamster lung fibroblasts (Kinsella and Radman, 1978). Another side, Tsuda and Takeda (1987) reported that 0.1 to 10  $\mu\text{g}/\text{ml}$  of TPA failed to increase the frequency of spots in the wings in flies exposed during larval development; however, the TPA's solvent, the form of larval exposure, and the cross employed then were different.

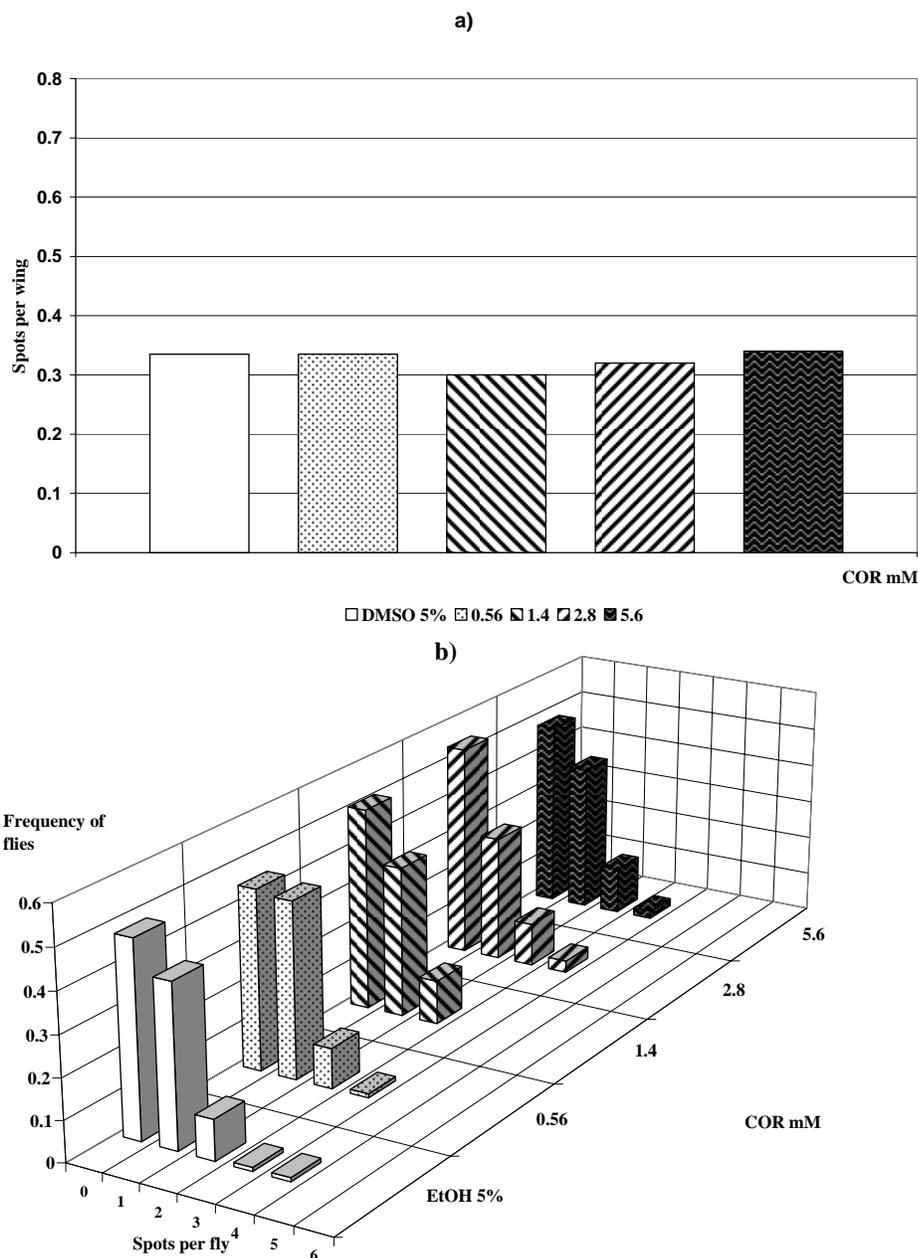


Figure 2. a) Frequency of total spots, and b) spots per fly distribution from flies exposed to COR.

The steroidal hormone COR was found negative in the SMART and affects slightly the SI of exposed flies. Results about the genotoxic activity of steroidal hormones are rather controversial

(Joosten *et al.*, 2004). In the present study we put cytotoxicity away as the explanation for the lack of effect based on the frequency of spots, which were similar in experimental and control series. That the presence of COR did not affect larvae development was confirmed through the distribution of the number of spots per fly, which was like that in the control series, too.

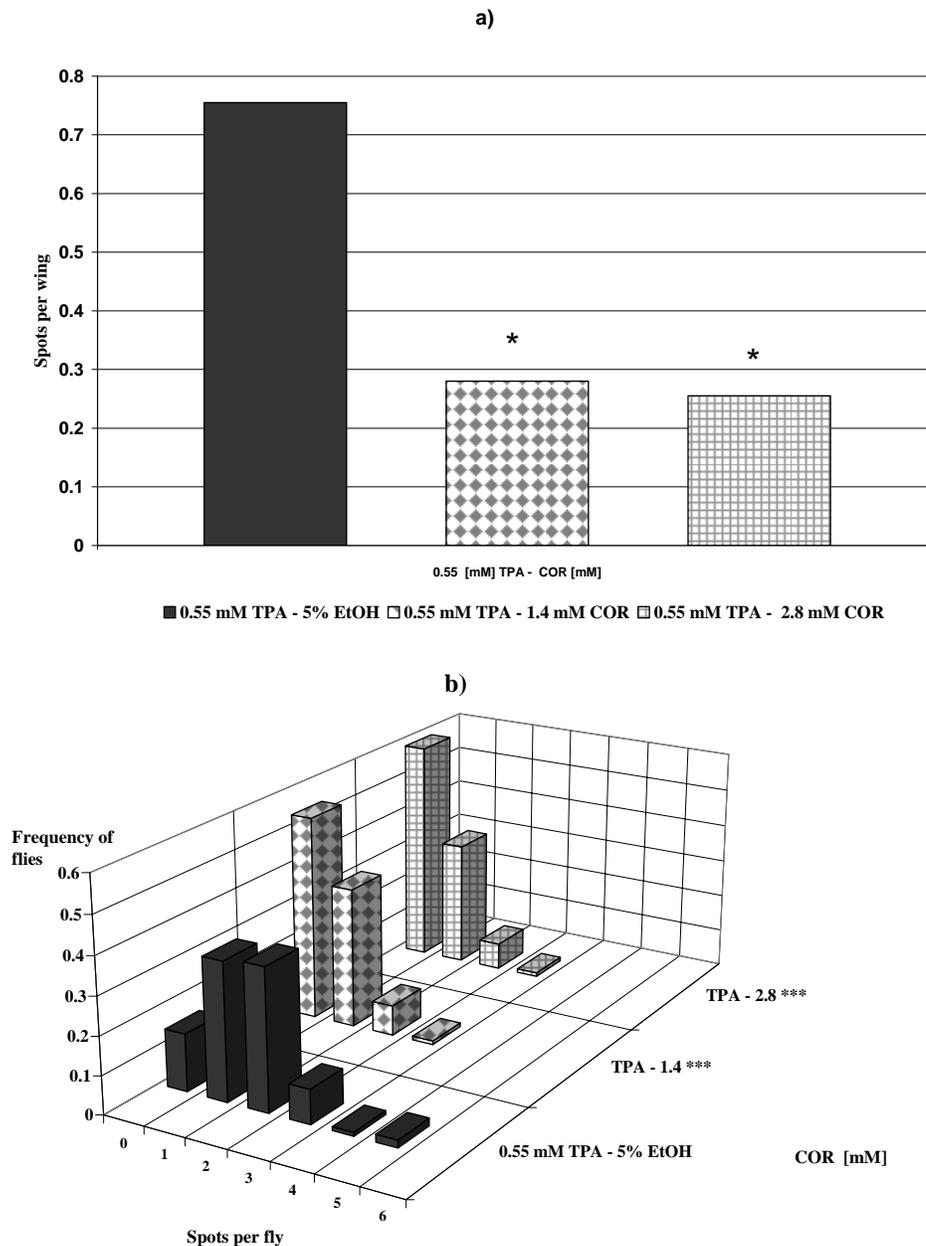


Figure 3. a) Frequency of total spots, and b) spots per fly distribution from flies exposed to TPA - COR.

In contrast, the SI from flies exposed to the combined treatment was higher, suggesting that COR diminished the TPA toxicity. The same was true for the frequency of spots on the wings, which were significantly lower after COR was given as the second part of the combined treatment. Again,

the distribution of the number of spots per fly confirmed that more spotless flies were recovered from TPA-COR treatments contrasting with the spotless flies recovered from TPA-EtOH (Figure 3b).

Like other organisms, in natural environments *Drosophila* could be threatened by parasitoids as the wasp *Leptopilina boulardi*. The innate cellular immune response associated with the formation of melanotic capsules around the eggs of the intrahemocoelic wasp is based partially on glucocorticoids (GCC) which regulate the larvae encapsulation capacity (high levels of GCC reduce the formation of capsules), but also is based in high levels of reactive intermediates of oxygen (ROIS) and nitrogen (RNIS), which are produced during encapsulation and have a role as cellular messengers, too. In summary, the defense response of *Drosophila* to parasitoid infection shows similar elements to those from the inflammation response in mammals (Carton *et al.*, 2002).

In the methodology used, TPA like the wasp infection could turn the production of pro-inflammatory cytokines on or increase the expression and activity of enzymes favoring ROS production (Fürstenberger *et al.*, 2006; Federico *et al.*, 2007). In *Drosophila*, some genes related to the immune response that are homologues to human genes have been already identified (Mackay, 2006; St. John and Xu, 1997), two genes for ODC activity (Rom and Kahana, 1993), seven different PLA<sub>2</sub>s genes (Valentin and Lambeau, 2000), genes with activity similar to COX and LOXs (Pages *et al.*, 1986), six PKC genes (Shieh *et al.*, 2002), and dNOS (Ray *et al.*, 2007). On the other hand GCC could inhibit the inflammatory reaction in several ways: interfering with PLA<sub>2</sub> and COX function (Samuelsson *et al.*, 2007), inducing the expression of anti-inflammatory genes (lipocortins), or in an indirect manner blocking pathways like NF- $\kappa$ B and the protein activator-1 (AP-1) (Pfahl, 1993). In concordance the GCC dexametasone inhibits the immune response against the parasitoids in *Drosophila*.

Our results showed that *Drosophila* and the SMART could be effective alternatives for the screening of anti-inflammatory activity from plant derivatives and metabolites with antioxidant capacity. In addition, this experimental procedure reduces in a significant manner the economic inversion for the screening of potential compounds and makes it possible to discriminate between anti-inflammatory (antioxidant) activity and the lack of response due to cytotoxicity of the treatment. More experimental work is needed to make accessible a concentration-effect curve focused to establish the biological meaning of treatments performed in rats, mice, and flies; but, this can be the first approach to the replacement of mammals for the screening of anti-inflammatory and antioxidant activities.

## Conclusion

The use of *Drosophila melanogaster* as a model for the characterization of the anti-inflammatory and antioxidant potential of compounds is reliable through the SMART because of the capacity of ROS to induce genetic damage and the similarity in the function of genes implied in the cellular immune response of *Drosophila melanogaster* and the inflammation reaction in mammals.

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