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Action of protoporphyrin-IX (PP-IX) in the lifespan of *Drosophila melanogaster* deficient in endogenous antioxidants, Sod and Cat

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ABSTRACT

Protoporphyirin-IX (PP-IX) is a precursor of the biosynthesis of the hemo group, most of the cytochromes and the chlorophylls. The PP-IX is used for medical purposes, and recently a report indicated that it exhibits a dual action since it can decrease or increase the genetic damage caused by N-nitroso-N-ethylurea (ENU) in somatic cells of *Drosophila*. PP-IX is known to be able to act as an anti- or pro-oxidant agent. The aim of the present research was to study the role of PP-IX on the lifespan of *Drosophila melanogaster*, taking into account the fact that increasing levels of ROS can accelerate the aging process. The *Canton-S* strain (*CS*) was used as well as *Sod* and *Cat* which are deficient in the endogenous enzymes, superoxide dismutase and catalase, respectively. Groups of females and males were treated separately with 5 mg/ml of PP-IX solution. The comparison of survival curves indicates that this pigment extended the lifespan of *CS*. In contrast, *Sod* strain showed that the opposite effect and had no effect in *Cat* strain. The fact that PP-IX reduces the mean lifespan in *Sod* deficient strain might suggest a pro-oxidant action of PP-IX, and consequently the cumulating of ROS as a superoxide could have a mutagenic effect as was shown recently. The results presented evidence of the dual effect of PP-IX.

Keywords: Protoporphyrin IX (PP-IX); Lifespan; Sod; Cat; Reactive Oxygen Species; Longevity; *Drosophila melanogaster*

1. INTRODUCTION

Aerobic metabolism and exposure to physical or chemical agents of anthropogenic origin generate reactive oxygen species (ROS) that are characterized by an unpaired electron. The excess of ROS in the cell causes oxidative stress which may damage DNA, RNA, proteins and lipids producing degenerative diseases, and may accelerate the normal process of aging [1]. Although many ROS are continuously generated during metabolism, neither cells nor their components are damaged due to the action of the physiological defense enzymes that include the following: superoxide dismutase (Sod), catalase (Cat) and glutathione peroxidase [2].

In addition, the cells can prevent oxidative stress through the action of compounds known as antioxidants that are defined as any substance which, when it is present in concentrations lower than those of an oxidizable substrate, delays or inhibits oxidation [3]. Since many antioxidants are found in food, their levels can increase. The compounds that have been more extensively studied for their antioxidant activity are ascorbic acid, α-tocopherol and β-carotene. These compounds have a similar structure which in most cases is responsible for their activity, and which includes at least an aromatic ring and one or more hydroxyl groups that can act as electron donors [4].

Studies *in vitro* as well as *in vivo* have shown that chlorophyll and its semi-synthetic derivative, sodium copper chlorophilin (SCC), are able to decrease or completely inhibit DNA damage induced by both physical [5] or chemical agents [6,7]. From the results obtained with different test systems, it was established that SCC works primarily through three mechanisms: a) as an antioxidant, inactivating free radicals, b) forming complexes with mutagens/carcinogens or their precursors, and c) inhibit-

ing the enzymes involved in the activation of carcinogens [8]. However, although most of the studies have provided evidence that SCC is an excellent antimutagen and/or anticarcinogen, some others have shown that this compound can increase DNA damage, either spontaneously or induced by other agents [9-11].

Previous studies demonstrated that SCC may act as promoter and inhibitor of the mutagenesis in *Drosophila*. In our study 48 h-old larvae were pretreated for 24 h with SCC or sucrose and then treated with chromium (VI) oxide (CrO_3), gamma rays, N,N-Dimethylhydrazine (DMH) or ENU immediately following completion of the pretreatment period (0-day delay) or delayed 1, 2 or 3 days. After delays of 0 and 1 day, clear evidence was found of a protective effect of SCC. Contrarily, after delays of 2 and 3 days the results showed a reversal effect, in other words that SCC-related genetic damage appeared more frequently than the events in the sucrose control suggesting a promoting effect. This dual effect of SCC with different agents suggested that it does not depend on the mechanism of action of the agent [10]. At present, evidence indicates that both the lower concentrations and the SCC metabolites such as PP-IX are involved in the mutagenic effect, and that copper could be responsible for antimutagenic activity [10].

Protoporphyrin-IX is a precursor of the hemo group, the chlorophylls and most of the cytochromes [12]. Some studies have provided evidence that the PP-IX can act as either pro-oxidant or antioxidant; for example, in the presence of light, PP-IX stimulates lipid peroxidation by Fe^{+2} and ascorbate [13] whereas lipid peroxidation is inhibited in darkness. Increased levels of Sod were also found in mice treated with PP-IX, suggesting that it induces the formation of superoxide radicals [14]. In rat strain CF1, Sod was rapidly induced after a single dose of PP-IX suggesting that superoxide radicals had been generated [14].

The most accepted hypothesis of aging was proposed by Gerschman [15] and Harman [1] who suggest that aging results from the accumulation of damage caused by free radicals; this is supported with evidence found by different systems [16-21]. The use of strains deficient in the expression of endogenous antioxidants has been practicable in testing the effect of dietary antioxidants in lifespan. *Drosophila melanogaster* has been extensively used to test the effect of dietary exogenous antioxidants in lifespan because it offers many advantages: its lifespan is short, 80 - 90 days (in laboratory conditions) and no mitotic divisions take place in the adult [22]. In addition, different mutant strains in genes involved in antioxidant defense such as catalase and superoxide dismutase enzymes could be used [16,20]. It is known that through the lifespan of *Drosophila* the activity of Cat decreases [18,23-25] however, the increased expression of the *Cat* gene does not extend the lifespan of flies, but rather protects against oxidative stress [26].

Some studies have shown that in both, mice [27] and *Drosophila* [28] a deficiency of the Mn-*Sod* gene (*Sod*2) severely reduces lifespan and increases the degree of oxidative stress which in turn, increases the levels of DNA damage and tumor incidence [29]. Unlike Cat, the over expression of *Sod*-Cu/Zn in *Drosophila* increases the lifespan [30,31] proportionally to the activity of the enzyme. Moreover, the simultaneous expression of Mn-*Sod* and *Sod*-Cu/Zn has an additive effect [32], whereas the simultaneous expression of the enzyme Mn-Sod and Cat does not provide additional benefits [33].

Since free radicals accelerate the aging process, the consumption of antioxidants has been promoted due to its ability to inactivate free radicals. Reports have shown that a concentration of 20 μg/mL of vitamin E, added in the culture medium of *Drosophila*, increased lifespan by 16% compared with the control group. However, adverse effects were also detected because its higher concentrations (200 μg/mL) reduced the lifespan significantly [34].

One of the best documented mechanisms of action of SCC is its activity as an antioxidant; it is known to be capable of preventing lipid peroxidation [35,36]. However, as mentioned earlier this compound, as well as PP-IX are capable of inducing genetic damage probably through generation of ROS [10,14]. To evaluate this possibility, and based on the fact that increased ROS levels accelerate the aging process, we defined our aim in this research as the assessment of the role of PP-IX in the lifespan of *D. melanogaster* strains deficient in CuZn-*Sod* and *Cat*.

2. MATERIALS AND METHODS

2.1. *Drosophila melanogaster* Strains

Canton-S wild type, *Sod* and *Cat* strains were used. The last two were obtained from the Bloomington Drosophila Stock Center. Sod and Cat enzymes constitute an evolutionary conserved ROS defense system against superoxide; Sod converts superoxide anions to H_2O_2, and Cat prevents free hydroxyl radical formation by breaking down H_2O_2 into oxygen and water.

Sod [n1] *red* [1]/TM3, *Sb* [1] *Ser* [1]: In *Drosophila*, deficiency of cytoplasmic CuZn-*Sod* (*Sod*1) in *Sod*1-null mutants imparts reduced lifespan, neurodegeneration, infertility, and hypersensitivity to further oxidative stress [37-39]. The gene encoding the enzyme is located in chromosome 3 [40]. Homozygotes have a significantly shorter mean and maximum lifespan than normal and are sensitive to paraquat, ionizing radiation and hyperoxia compared to control flies.

Cat [n1]/TM3, *Sb* [1] *Ser* [1]: This strain is deficient in the enzyme Cat, the gene encoding the enzyme is also

found in chromosome 3, in the region 44.3 [40]. Cat enzyme is involved in the decomposition of H_2O_2 generated during cellular metabolism [41]. Only about 10% of homozygous *Cat* [n1] flies eclose from their pupal cases. Homozygous *Cat* [n1] mutants also show a reduced lifespan, living half as long as wild type flies [42].

Three groups of 25 males and virgin females 16 h old were placed separately for 24 h in homeopathic vials (10.5 × 2.4 cm) containing 0.7 mg of *Drosophila* instant medium (Formula 4 - 24 Carolina Biological Supply Co.) with 2.5 mL of tap water or 5 mg/mL solution of PP-IX. In all, 100 to 225 females or males were tested. Three experiments were performed for each group (0 and PP-IX). Twice a week dead flies were counted, and live individuals were transferred to vials with freshly corresponding treatment. The PP-IX disodium salt (CAS 50865-01-5) was purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Statistical Analysis

The Kaplan-Meier test was used to obtain the cumulative survival curves for each experimental group, and for each sex. Data for survival were plotted and curves were compared using the Wilcoxon test (XLSTAT software) [43]. The General Linear Model (GLM) was used to determine any interactions between sex, strains or treatment.

3. RESULTS

Based on the toxicity test in which three concentrations for PP-IX were tested (0.5, 5 and 50 mg/mL.), we found that the 5 mg/mL concentration was appropriate for chronic treatment in adults. The mean lifespan (MLS) and maximum lifespan (ML) for each sex and treatment were evaluated. MLS is the time where half of the treated individuals died and ML is the total lifespan of individuals.

Table 1 shows the values of both traits of each tested strain. The analysis of variance (ANOVA) at 95% confidence showed significant differences among strains with a relation: *Sod* < *Cat* < *CS* (see **Figure 1**.). The treatment with PP-IX significantly prolonged the MLS of females (by 17.4 days) and males (by 9.7 days.) in *CS* as well as *Cat* (by 1.6 and 3.2 days for females and males, respectively.); however, in the latter strain no noteworthy differences were found. In contrast, PP-IX shortened the MLS significantly in both sexes of *Sod* strain, where the females exhibited a shorter MLS (12 days.) than males (7 days.). Regarding the ML, PP-IX reduced it significantly only in the *Sod* strain; the reduction of ML was different between females (25 days less.) and males (11 days less.) with respect to the control. No significant differences were found between sex in *CS* and *Cat* strains.

Figure 2 shows the survival curves for each strain separately by sex after chronic treatment with PP-IX; data corresponding to females are given in the left panel and for males in the right one. Taking together data for females and males, the results reflect that PP-IX increases the MLS by 13.5 days (26%) in *CS*, by 2 days (5%) in *Cat* but reduces it in *Sod* by 10 days (26%) with respect to the control.

4. DISCUSSION

Longevity is a quantitative trait, with continuous phenotypic variation attributable to the joint segregation of

Table 1. Effect of chronic treatment with 5 mg/ml of PP-IX on the lifespan of *CS*, *Sod* and *Cat* strains.

Strain	Sex	Chronic Treatment PP-IX (5 mg/mL)	n	MLS (days) ± *SE*	ML [days]	*P* value (0 *vs.* *PP-IX*)
CS	♀	0	225	50.7 ± 2.4	84	
		PP-IX	150	68.1 ± 3.0	84	<0.0001
	♂	0	225	53.3 ± 2.4	84	
		PP-IX	150	63.0 ± 0.1	84	<0.0001
Sod	♀	0	200	41.8 ± 2.6	70	
		PP-IX	125	29.6 ± 3.3	45	<0.0001
	♂	0	200	36.2 ± 2.6	49	
		PP-IX	125	29.0 ± 3.3	38	<0.0001
Cat	♀	0	100	44.9 ± 3.7	59	
		PP-IX	150	46.5 ± 3.0	63	n.s.
	♂	0	125	43.2 ± 3.3	63	
		PP-IX	150	46.4 ± 3.0	66	n.s.

n= Number of individuals tested, MLS = Mean lifespan and ML = Maximum lifespan. The MLS were calculated from the general linear model (least square means). P values were obtained from comparisons between the survival proportion curves. The confidence intervals of MLS were obtained from the Kaplan-Meir analysis.

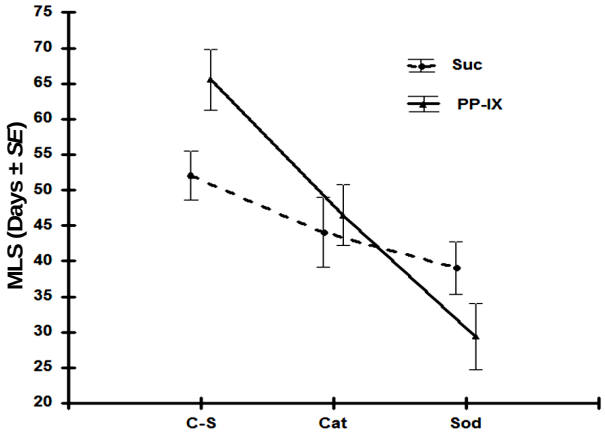

Figure 1. Mean lifespan (MLS) relationship between strains.

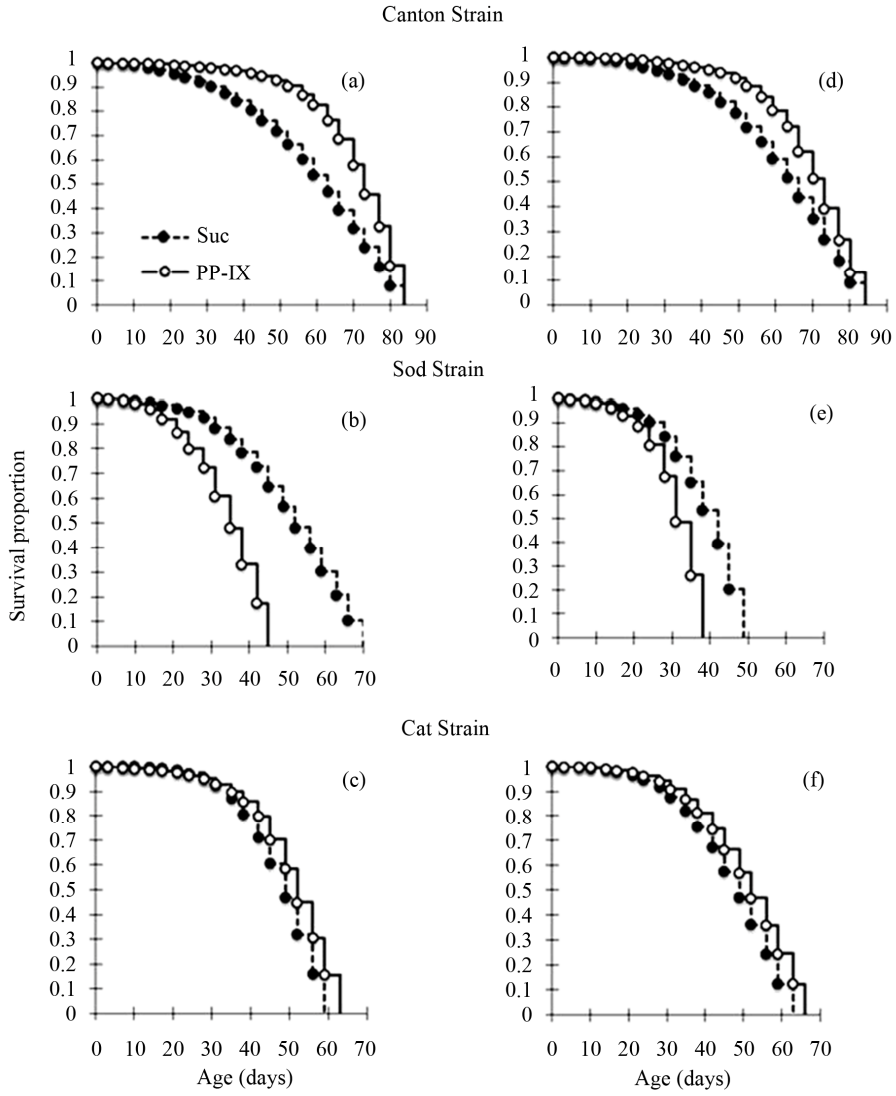

Figure 2. Represents the survival for females (a), (b), (c) and for males (d), (e), (f) Greenwood confidence intervals were employed. Data for survival were plotted and curves were compared using the Wilcoxon test (XLSTAT software) (Addinsoft 2011). Log-rank, Wilcoxon and Tarone-Ware analysis showed a significant difference ($p < 0.0001$) when treatments PP-IX and control were compared for each sex of strain. Probability level was: alpha = 0.05.

multiple interacting quantitative trait loci and with effects that are highly sensitive to the environment. *D. melanogaster* has been a historically important system for investigating the genetic basis of longevity, which relies on two sources: its powerful genetic tool as a model system, and a natural ecology that provides substantial genetic variation across significant environmental heterogeneity.

In a previous study PP-IX was shown to be able to act as an antimutagen immediately after being administrated to the flies and as a mutagen a few days later [10]. One possible explanation of this effect is that PP-IX provokes damage through generation of ROS. The present study showed that in *Sod*-deficient strain, chronic treatment with PP-IX reduced the MLS by 12 days for females and by 7 days for males. The ML was shorter by 25 days for females and by 11 days for males. Nevertheless, neither MLS nor ML was affected in *Cat*-deficient individuals.

These results are in accordance with those reported by Lebovitz *et al.* [37] in mice and by Duttaroy [28] in *Drosophila* who showed that the deficiency in the mitochondrial Mn-*Sod* gene significantly reduces life expectancy due to the increase of the superoxide radical. The same was observed in the absence of the gene encoding the cytosolic CuZn-Sod, but the effect was not as severe as when a deficiency of mitochondrial Sod occurs. It is

likely that under those conditions PP-IX produces no action on the superoxide generated by the radiation, but probably acts as a pro-oxidant generating superoxide as demonstrated by Afonso *et al.* [14].

Another example that supports this hypothesis is that PP-IX has been used for several years in photodynamic therapy [14,44-46]. This is so due to its ability to induce superoxide radicals that react with molecular oxygen producing peroxide radicals which cause lipid peroxidetion, and leading to different cell damages such as structural changes of the cell membrane, damage to proteins, inactivation of receptors, enzymes and ion channels, all of which can lead to cell death [47].

The protective activity of PP-IX observed in the *CS* strain in this study could be the result of the elimination of free radicals, particularly H_2O_2. Afonso *et al.* [14] found that Cat activity increased 30% in rats of the strain CF1, 2 h after the injection of PP-IX, with the decrease in H_2O_2 levels. Although the results obtained with *Cat* strain showed a minimum increase in the MLS and ML, in the wild type strain its effect was more evident; PP-IX increased the MLS by 26% compared to the control. However, a report indicated that the over expression of Cat by 80% did not increase the lifespan of *Drosophila*, but protected it against oxidative stress [48]. Furthermore, Griswold *et al.* [49] molecularly characterized six alleles and found that *Cat* [n2], *Cat* [n3], *Cat* [n5] and *Cat* [n6] reduced the Cat activity, viability and normal longevity, but the alleles *Cat* [n1] and *Cat* [n4] showed no activity of Cat, yet longevity and viability were reduced. Mackay *et al.* [50] showed that *Cat* null allele caused a significant increase in the frequency of spontaneous mutations. The increase in MLS in *CS* and *Cat* could be attributable to the antimutagenic effect reported by other authors [5, 51-53], who suggest that the protective effect of both PP-IX and SCC, (porphyrin bound to Cu^{+2}.) is due to their ability to arrest free radicals generated by radiation and to increase the expression of endogenous antioxidants [54].

Although the PP-IX treatment reduced the MLS in the Sod-deficient strain (Probably because of its pro-oxidant action inducing the formation of O^{2-} radicals, the H_2O_2 produced by this increase in superoxide.), the antioxidant action of PP-IX could be added to the action of glutathione peroxidase enzyme which can also reduce H_2O_2.

According to the effect of PP-IX on both the wild type *CS* and the *Cat* strain, we suggest three possible mechanisms of action: a) as inductor of the antioxidant enzymes to achieve normal levels of activity; b) as an antioxidant, reducing the levels of peroxide; and c) both actions occurring simultaneously in the organism provoking an increase in the lifespan of the treated organisms. The *CS* strain is the best evidence of the c) mechanism, since in this strain PP-IX delayed death and strongly increased MLS at birth in both sexes by 13.5 days.

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