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SHORT REPORT

A Further Study of the Role of Copper in Regard to the Antimutagenic Action of Sodium Copper Chlorophyllin (SCC) in Somatic Cells of *Drosophila melanogaster*

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Abstract: Previous findings suggest that copper plays a crucial role in the antimutagenic effect of sodium copper chlorophyllin (SCC). The objective of the current research was to compare the antimutagenic effects of two SCC compounds with different amounts of copper (3.7% and 5.4%, respectively) on the genetic damage induced by gamma rays in somatic cells of *Drosophila*. Data indicate that an increase in copper content of 31.5% in SCC-5.4 resulted in a greater inhibition of gamma ray genetic damage of 49% whereas only a 2% inhibition with SCC-3.7 occurred. Of greater interest is the association of SCC with a variety of uses in humans, such as a chemo preventive agent and food supplement. A greater attention to the concentration of copper in the SCC product in use should be required.

Keywords: copper, chlorophyllin, gamma ray mutagenesis, *Drosophila,* somatic mutation

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Introduction

Sodium copper chlorophyllin (SCC) exhibits potent antimutagenic and anticarcinogenic activity against many agents[1] including gamma rays.[2–4] One of its mechanisms being the scavenging of free radicals as an antioxidant.[5,6] Contrarily, other results have shown that SCC may both inhibit and promote the genetic effect of some agents.[7–10] Most recently Tumolo and Lanfer-Marquez[11] published a review of the dual effects of SCC.

In a recent study employing somatic cells of *Drosophila*, evidence was found suggesting the possibility that the dual effect of SCC could indicate the effects of the dissociation products of the protoporphyrin-copper complex. A progressive accumulation of a porphyrin ring, such as the protoporphyrin IX (PP-IX), provoked a mutagenic effect and the Cu^{+2} demonstrated inhibitor action. The inhibitory activity of Cu^{+2} (in the form of $CuCl_2$) during all the times monitored, suggested that the presence of Cu^{+2} in SCC is strongly related to its prolonged antimutagenic effect.[12]

The exact mechanisms of SCC to serve as a mutagen/carcinogen are still not known. In view of these findings, however, it is important to investigate the conditions under which SCC behaves as antimutagen or mutagen, including the composition of the avaible commercial presentations. Commercial grade SCC is a mixture of hydrolized chlorophyll derivatives including CuCle4, CuCle6, copper pheophorbide, a copper rhodin g7, and their degradation products.[13] The objective of the current research was to compare the antimutagenic effects of different amounts of copper in two commercial sodium copper chlorophyllin forms on the genetic damage induced by 15 Gy of gamma rays.

Material and Methods

In order to monitor the potential antimutagenic effect of SCC-3.7 and SCC-5.4, the wing spot test was used. The *Drosophila* wing somatic mutation and recombination test (SMART) detects the potential of an agent to induce loss of heterozygosity resulting from gene mutation, chromosomal rearrangement, or chromosome breakage. The test uses the wing-cell recessive markers multiple wing hairs (*mwh*, 3–0.3) and flare (*flr*^{3}, 3–38.8) in transheterozygous *mwh +/+ flr*^{3} individuals. When a genetic alteration is induced in a mitotically dividing cell of a developing imaginal wing disc, it may give rise to a group of mutant cells (spots) easily recognizable on the adult wing blade. For a description of the mutants see Lindsley and Zimm[14]. Three-day-old *mwh/mwh* females and *flr*^{3}/In(3 LR)TM3 *Ser* males were allowed to mate for 2 h and then transferred to culture bottles with fresh food. Twelve bottles with 100 couples in each were used. Oviposition was restricted to a 2 h period in order to obtain more homogeneous samples of adults of similar age being tested. Then, 48h-old larvae were collected by density gradient using a 20% sucrose solution. They were then washed with 24 °C ± 1 °C tap water and pretreated for 24 h in the dark, in flasks (1/4 L) with a paper filter (Whatman # 2), saturated with 69 mM SCC-3.7, SCC-5.4 or 5% sucrose (SUC) as a negative control. SCC-3.7 and SCC-5.4 were dissolved in a 5% sucrose solution. Distilled water was used for all solutions. It is worth noting that Sigma Chemicals Company distributes SCC with 3.7%, 3.87%, and 5.4% of Cu^{+2}, and 5.77%, 6.07%, and 6.5% of Na, respectively. The SCCs extreme in percentages of Cu^{+2} were selected for this study. Both SCC stocks were purchased from Sigma Chemicals Company (St. Louis, MO). The lot number for SCC-3.7 was 77H0594 and for SCC-5.4%, 74H0067.

On completion of the pretreatment period, larvae were washed with tap water at 25 °C ± 1 °C. Larval-adult viability was measured by an indirect method to determine whether the different SCC caused cell death. Aliquots from each pretreatment exposed or not with 15 Gy gamma rays, were placed in vials containing 1.5 g of *Drosophila* instant medium (formula 4–24 Carolina Biol. Supply Co. Burlington, North Carolina) with distilled water. Three experiments were carried out.

Upon eclosion, flies were fixed with 70% alcohol and the wings of the *mwh +/+ flr*^{3} flies (ie, non-*Ser*) were mounted on slides for 400X microscopic analysis. The wings were examined to identify small single spots (one or two cells), large single spots (more than two cells) of either *mwh* or *flr*, and *mwh-flr* twin spots. Briefly (1) single mwh spots are inferred to arise from point mutations or deletions at the wild type allele of the locus or mitotic recombination in the chromosomal region between *mwh* and *flr* locus, (2) single *flr* spots from mutation/deletion at the *flr*+ locus or double interchange, and (3) twin spots arise

following a recombination event between flr and the centromere.[15] Comparisons were made between various classes using the SMART statistical diagnosis proposed by Frei and Würgler[16] in order to determine differences between treatments.

Results

None of the two samples of SCC was toxic at the concentration used in this experiment of viability (SCC-3.7 had 89% ± 0.2% and SCC-5.4, 87% ± 0.3%) and were not different with respect to control viability (90% ± 0.4%). Table 1 provides the results of the experiments described above. Comparisons with SMART diagnosis between SCC-3.7 or SCC-5.4 with their common control, as well as between SCC-3.7 and SCC-5.4, showed no evidence of statistical differences, except in twin spot frequency in which there was one spot more in SCCs. The result was inconclusive when they were compared with the SUC control. However, upon comparing SCC-3.7 + 15 Gy and SCC-5.4 + 15 Gy with the irradiated control, we found significant reductions in the frequencies in all classes of spots from both SCC, except for small spots from the SCC-3.7 series. It is worth noting that although a significant reduction of genetic damage was observed with both SCCs in respect to that induced by 15 Gy of gamma rays, the differences were weak for SCC-3.7 and positive for SCC-5.4, except for large spots in which statistical diagnosis was weak positive. A comparison related with the percentage of Cu^{+2} in SCC-3.7 with respect to SCC-5.4 indicates that a 31.5% increase in copper amplifies the effectiveness of SCC at inhibiting the effects of gamma rays from 23% with SCC-3.7 to 49% with SCC-5.4.

Discussion

The crucial role of copper in the antimutagenicity of porphyrins was demonstrated earlier by Arimoto,[17] who found that the metal-free porphyrins in *Salmonella* had only a minor antimutagenic effect as compared with those in which an appropriate metal was present. The importance of the nature of metal on the porphyrin ring was demonstrated by Lanfer,[18] who compared the antioxidant activity of six natural isolated chlorophyll derivatives: chlorophyll a and b, pheophytin a and b, pheophorbide a, and the synthetic Cu-chlorophyllin. It was found that Cu-chlorophyllin presented a higher antioxidant activity than that of natural chlorophylls. As indicated above, the presence of Cu^{+2} in SCC plays an important role in SCC antimutagenesis.[12]

The present data indicate that a 31.5% increase in copper in SCC-5.4, when compared to SCC-3.7, provoked a reduction of 67% in small, 50% in large, and 80% in twin spots. This represents more than twice the reduction obtained for SCC-3.7 (23% and 49% respectively). These findings are supported by Ferruzzi's results.[19] Ferruzzi examined the stability of SCC during simulated gastric and small intestinal digestion of SCC *in vitro* and found that one of the SCC components, Cu-chlorin e4, has digestive stability.

Table 1. The mutation frequency induced in 48 h *mwh +/+ flr³ D. melanogaster* larvae pretreated with two different commercial SCC compounds differing in the percentage of copper (Cu^{+2}) and exposed to 15 Gy of gamma rays.

Treatment	No. of wings	Spots				% of reduction
		Spots per wing (number of spots)				
		Small single spots	Large single spots	Twin spots	Total spots	
		(1–2 cells) m = 2	(>cells) m = 5	m = 5	m = 2	
SUC	120	0.27 (36)	0.06 (7)	0.025 (3)	0.35 (42)	
SCC-3.7% Cu^{+2}	120	0.27 (32)$^{-}$	0.07 (8)$^{-}$	0.03 (4)^{i}	0.37 (44)$^{-}$	
SCC-5.4% Cu^{+2}	120	0.28 (34)$^{-}$	0.05 (6)$^{-}$	0.03 (4)^{i}	0.37 (44)$^{-}$	
SUC+15 Gy	120	1.02 (123)	2.70 (324)	0.78 (94)	4.51 (541)	
SCC-3.7+15Gy	120	1.04 (125)$^{-}$	2.00 (238)^{w}	0.43 (52)^{w}	3.46 (415)^{w}	23%
SCC-5.4+15Gy	120	0.40 (40)$^{+}$	1.72 (207)^{w}	0.16 (19)$^{+}$	2.28 (274)$^{+}$	49%
SCC-3.7 vs. SCC-5.4		+	–	w	w	26%

Notes: The table expresses the mean of three experiments. Statistical diagnoses according to Frei and Wüergler (1988): + = positive; – = negative; w = weak positive; i = inconclusive; m = multiplication factor. Probability levels: alpha = beta = 0.05. One side statistical tests. The % of reduction column shows the percentage of genetic damage reduction with respect to Suc+15 Gy group.

It was also found by Ferruzzi that the Cu-chlorin e6, representing a lower amount from the SCC components, was digestively labile and that both components can be absorbed by intestinal cells. These data could indicate that the excess copper in SCC-5.4 is able to inactivate additional free radicals and in this way induce a greater reduction in genetic damage induced by gamma rays. It was suggested that copper could associate with proteins and participate in anabolic homeostatic pathways.[6]

Finally, the extent of the inhibitory effect of SCC reported in different publications[11] may have resulted from differences in the copper content of commercial SCC compounds. The extended use of SCC in several products and its relationship with possible health benefits has also been the subject of several studies because of its antimutagenic, anticarcinogenic, and antioxidant activities. However, as mentioned earlier, its mechanisms of action are not yet well understood and some studies indicate that it can serve as mutagen and carcinogen. Recently Campos[20] reported that chlorophyllin significantly reduced cell survival in the adenocarcinoma cell line HT29. In view of those finding, and due to the association of SCC with a variety of uses in humans, perhaps notably as a chemo preventive agent and food supplement, this should demand, if not in practice as yet, greater attention to the different concentration of copper in the SCC products in use.

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Author Contributions
Conceived and designed the experiments: EP. Analyzed the data: EP, MC, SZ. Wrote the first draft of the manuscript: EP. Contributed to the writing of the manuscript: EP, MC, SZ. Agree with manuscript results and conclusions: EP, MC, SZ. Jointly developed the structure and arguments for the paper: EP, MC, SZ. Made critical revisions and approved final version: EP, MC, SZ. All authors reviewed and approved of the final manuscript.

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