Investigation of Chlorophyllin in CHO-k1 (wild-type) and CHO-xrs5 (repair-deficient) Cells by a Chromosomal Aberration Assay

Priscilla D. Negraes¹, Barenice Q. Jordão¹, Veronica E. P. Vicentini² and Mávio S. Mantovani^{1,*}

¹ Departamento de Biologia Geral, Universidade Estadual de Londrina, PR, Brazil

² Departamento de Biologia Celular e Genética, Universidade Estadual de Maringá, PR, Brazil

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Summary Chlorophyllin, a sodium-copper salt derivative of chlorophyll *a* and *b* was evaluated at concentrations of 6.25, 12.5 and 25 $\mu\text{g/ml}$ with regard to its clastogenic and anticlastogenic potential in Chinese hamster ovary cells CHO-k1 (wild-type) and CHO-xrs5 (DNA repair-defective), using a chromosomal aberration test. Cells were subjected to continuous exposure for 12 h to chlorophyllin in the presence or absence of ethyl methane sulfonate (EMS, 310 $\mu\text{g/ml}$). The results demonstrate that under the experimental conditions utilized, chlorophyllin was not cytotoxic nor clastogenic in either of the cell systems employed. Moreover, anticlastogenic activity was also not observed.

Key words Chlorophyllin, CHO cells, Chromosomal aberrations.

Currently, there is a growing need to test synthetic and natural chemical compounds, which are in great numbers in our environment, for possible carcinogenicity and mutagenicity. In addition, many substances of plant origin are being investigated with regard to their antimutagenic/anticarcinogenic potential (Mitscher *et al.* 1992).

Therefore, chlorophyllin, a sodium-copper salt derivative of chlorophyll, has been studied for its protective action against the carcinogenic effects of various physical and chemical agents, and how it relates to the mutagenic and clastogenic activity of genotoxic agents. Reported studies suggest that the effects of these compounds correlate with the concentrations used, however without any dose-response type relationship (Romert *et al.* 1992).

Therefore, in view of the ambiguity with respect to the mechanism of action of chlorophylls and their derivatives in the protection against as well as in the induction of DNA damage, the aim of the present study was to evaluate chlorophyllin for clastogenicity and anticlastogenicity in wild-type (k1) and DNA repair-defective (xrs5) CHO cells using a chromosomal aberration test.

Materials and methods

Cell lines and culture conditions

Wild-type Chinese hamster ovary (CHO-k1) cells and a variant line defective in the repair of double-strand DNA (CHO-xrs5), furnished by the Mutagenesis Laboratory of USP-Ribeirão Preto (SP)/Brazil, were grown as monolayers in 25 cm² culture flasks. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12, supplemented with 10% fetal bovine serum and 0.1% antibiotic-antimycotic solution, in a BOD chamber at 37°C. Under these conditions, the cell cycle was 12 h.

* Corresponding author, e-mail: biomsm@uel.br

Chemical agents

Chlorophyllin (Sigma) was used at final concentrations of 6.25, 12.5 and 25 $\mu\text{g/ml}$ in culture medium, and ethyl methanesulfonate (EMS) (Acros Organics) at 310 $\mu\text{g/ml}$, both diluted in PBS (Ca^{2+} and Mg^{2+} free, pH 7.4). The concentrations employed were determined based on pilot experiments.

Chromosomal aberration test

Cells were cultivated for a period of one cell cycle (12 h) in flasks (25 cm^2) containing complete medium, and cells were then treated with EMS with and without chlorophyllin for 12 h. Metaphasic cells were obtained employing the technique described by Preston *et al.* (1981), and colcemide (0.1 $\mu\text{g/ml}$ Demecolcine, Sigma) was added to the cultures 1 h prior to harvesting. Cells were washed twice with PBS (Ca^{2+} and Mg^{2+} free, pH 7.4), trypsinized (0.025%) and then subjected to hypotonic treatment for 20 min in 1% sodium citrate at 37°C, with agitation every 5 min. Cells were fixed with methanol : acetic acid (3 : 1), and slides were prepared and stained with 5% Giemsa. The results were from 3 independent experiments.

Slide analysis

The mitotic index was calculated as the number of existing metaphases per 1000 cells. For the evaluation of structural chromosomal aberrations (breaks), 100 well-spread metaphases were analyzed in a blind test for wild-type CHO-k1 cells and 50 metaphases for the mutant cell line CHO-xrs5. Chromosomal alterations were classified as chromatid-type (ct) and isochromatid-type (ic), in addition to quadriradial (qr) and triradial (tr) figures, centric and acentric rings (cr, ar), simple acentric fragments (saf), double acentric fragments (daf) and dicentric chromosomes (d). Rearrangements involving up to 2 chromosomes were described as simple (sr) and more than 2 chromosomes as complex (cr). Metaphases with more than 5 aberrations were identified as having multiple alterations (ma).

Statistical analysis

The data on the total number of cells with chromosomal aberrations were analyzed by the conditional test for rare event detection (Pereira 1991) at significance level of $\alpha=0.05$.

Results

Table 1 presents the mitotic indices and the number of cells bearing alterations, which were obtained for each of the different treatments conducted with both wild-type cells and mutant cells. The types of chromosomal aberrations found are shown in Table 2 and Fig. 1.

Statistical analysis demonstrated that only in CHO-xrs5 cells were treatments containing EMS cytotoxic. None of the concentrations of chlorophyllin used in either of the cell lines studied were clastogenic under the conditions employed, since the values did not differ statistically from those found for the negative control. Moreover, no protective effect was observed, since in CHO-xrs5 as well as CHO-k1 cells treated with EMS and chlorophyllin simultaneously, the total number of aberrant cells was not significantly distinct from that resulting from the treatment of cells with only EMS (positive control).

Discussion

In the present study, 3 concentrations of chlorophyllin (6.25, 12.5, 25 $\mu\text{g/ml}$), alone or combined with the DNA damage-inducing agent EMS, were tested for possible clastogenic and anticlastogenic effects. In order to provide for an even better understanding of the mechanism of action of

Table 1. Mitotic index and frequency of cells bearing chromosomal aberrations in cultures submitted to continuous exposure to chlorophyllin and ethyl methane sulfonate

Treatments	Concentrations ($\mu\text{g/ml}$)	Mitotic index ¹ (%)	Total number of aberrant cells ²
CHO-k1			
Control		4.60	12
EMS	310	4.00	43*
Chln	6.25	4.27	8
	12.5	4.80	16
	25	4.80	15
Chln+EMS	6.25	4.33	41
	12.5	4.47	30
	25	4.13	35
CHO-xrs5			
Control		3.23	27
EMS	310	2.00*	73*
Chln	6.25	2.60	19
	12.5	2.73	29
	25	2.57	23
Chln+EMS	6.25	2.03*	66
	12.5	1.90*	75
	25	1.80*	70

Ethyl methanesulfonate (EMS), chlorophyllin (Chln), * statistically significant, $0.0001 < p < 0.05$. ¹ 3000 cells. ² 300 CHO-k1 cells, 150 CHO-xrs5 cells.

Table 2. Types of chromosomal aberrations found in anticlastogenicity studies of chlorophyllin in cells continuously exposed to chlorophyllin and ethyl methane sulfonate

Treatments	Concentrations ($\mu\text{g/ml}$)	Types of aberrations												Total
		ct	ic	qr	tr	daf	saf	d	ar	cr	sr	cr	ma	
CHO-k1														
Control		11	0	0	0	3	0	1	0	1	0	0	0	16
EMS	310	31	9	0	1	3	4	0	0	1	0	1	1	51
Chln	6.25	2	0	0	0	1	2	3	0	1	0	0	0	9
	12.5	11	0	0	0	2	2	2	0	1	0	0	0	18
	25	7	2	0	0	2	1	1	0	4	1	0	0	18
Chln+EMS	6.25	30	2	1	5	11	4	0	0	0	1	0	0	54
	12.5	21	5	0	3	7	0	1	1	0	1	0	0	39
	25	27	3	2	3	1	1	0	0	1	0	0	0	38
CHO-xrs5														
Control		24	1	0	0	1	6	1	0	0	0	0	0	33
EMS	310	89	1	2	3	0	11	1	0	0	2	0	2	111
Chln	6.25	15	0	0	0	0	4	2	0	0	0	0	0	21
	12.5	26	0	0	0	0	8	1	0	0	0	0	0	35
	25	16	0	0	0	1	10	0	0	0	0	0	0	27
Chln+EMS	6.25	83	2	0	3	1	13	0	0	0	0	0	1	103
	12.5	97	3	0	4	2	9	0	0	0	0	0	1	116
	25	90	2	1	5	1	6	0	0	1	0	0	0	106

Ethyl methanesulfonate (EMS), chlorophyllin (Chln), chromatid (ct), isochromatid (ic), quadriradial (qr), triradial (tr), double acentric fragment (daf), simple acentric fragment (saf), dicentric (d), acentric ring (ar), centric ring (cr), simple rearrangement (sr), complex rearrange (cr), multiple alterations (ma).

Fig. 1. Chromosomal aberrations in CHO-k1. A) dicentric, B) isochromatid break, C, D) chromosomal rearrange, E) ring.

this compound, the study was conducted not only in a wild-type cell line (CHO-k1), but also in a DNA repair defective mutant line (CHO-xrs5).

Chlorophyllin, at the concentrations used, did not demonstrate any clastogenicity in either of the cell systems employed, in concordance with *in vitro* results previously reported by Bez *et al.* (2001a, b) and Rampazo *et al.* (2002).

CHO-xrs5 cells do not produce the protein Ku80, involved for example in NHEJ (non-homologous end joining) repair (Johnston *et al.* 1998), and are therefore defective in the repair of breaks in double-strand DNA. This type of cell is susceptible to X-radiation, and also exhibits cross-sensitivity to bleomycin and alkylating agents. The results presented are thereby similar to cytogenetic studies where mutant cell lines defective in the repair of double-strand breaks have been shown to have a greater number of spontaneous chromosomal aberrations and a much higher frequency of induced aberrations when compared with wild-type cells, mainly chromatid type aberrations (Stopper *et al.* 1997). Therefore, it was likely genotoxicity that was observed for this cell system in treatments where EMS was present.

Although chlorophylls and their derivatives have been extensively investigated for their potential as antigenotoxic, anticlastogenic, antimutagenic and anticarcinogenic agents in various test systems (Renner 1990, Ghosh *et al.* 1991, Sen *et al.* 1991, Abraham *et al.* 1994, Sarkar *et al.* 1994, Edenharder *et al.* 1995, Negishi *et al.* 1997, Pimentel *et al.* 1999, Bez *et al.* 2001a, b, Rampazo *et al.* 2002), other studies have demonstrated that chlorophyllin as well as chlorophyll can show genotoxic, clastogenic and carcinogenic effects (Romert *et al.* 1992, Sarkar *et al.* 1994, 1996). Romert *et al.* (1992), working with different systems, observed that low concentrations of chlorophyllin potentiated twofold the mutagenicity of tobacco-specific nitrosamines, NNN and NNK, in *Salmonella* and the V79 assay. However, these investigators showed that high concentrations of chlorophyllin blocked the mutagenicity of the nitrosamines, thus suggesting that chlorophyllin can act as a positive or negative modifier of mutagenicity.

Once the metabolic activation of chlorophylls or chlorophyllin does not appear to be involved in their ability to induce or protect against DNA damage (Gentile and Gentile 1991), the efficacy of their antimutagenicity, in addition to being related to the type and origin of the mutagen, should depend on the test system, concentration used and inducing agent employed, as proposed by Bez *et al.* (2001b). Our findings lend support to this notion, since there was no evidence of any protective effect for chlorophyllin under the experimental conditions described.

The absence of anticlastogenic activity in CHO-xrs5 cells may suggest that under particular conditions, chlorophyllin displays a bio-antimutagenic protective mechanism where some steps pertaining to the repair of double-strand DNA are involved.

Moreover, it should be noted that even when chlorophyllin was tested in repair-defective cells, there was no significant increase in the number of induced chromosomal aberrations, suggesting that there is no risk in the utilization of this compound. Further studies should be developed in relation to the concentrations and exposure times of chlorophyllin to provide new insights into its promising use in protecting against the mutagenicity of various compounds, environmental or otherwise.

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References

- Abraham, S. K., Sarma, L. and Kesavan, P. C. 1994. Role of chlorophyllin as an *in vivo* anticlastogens: protection against gamma-radiation and chemical clastogens. *Mutat. Res.* **322**: 209–212.
- Bez, G. C., Jordão, B. Q., Vicentini, V. E. P. and Mantovani, M. S. 2001a. Investigation of genotoxic and antigenotoxic activities of chlorophylls and chlorophyllin in cultured V79 cells. *Mutat. Res.* **497**: 139–145.
- , —, — and — 2001b. Differential protection of A and B chlorophyll in MMC-mediated induction of chromatid and isochromatid breaks in human lymphocytes culture. *Cytologia* **66**: 313–318.
- Edenharder, R., Leopold, C. H. and Kries, M. 1995. Modifying actions of solvent extracts from fruit and vegetable residues on 2-amino-3-methylimidazo[4,5]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5]quinoxaline (MeIQx) induced mutagenesis in *Salmonella typhimurium* TA 98. *Mutat. Res.* **341**: 302–318.
- Gentile, J. M. and Gentile, G. J. 1991. The metabolic activation of 4-nitro-*o*-phenylenediamine by chlorophyll-containing plant extracts: The relationship between mutagenicity and antimutagenicity. *Mutat. Res.* **250**: 79–86.
- Ghosh, A. K., Sen, S., Sharma, A. and Talukder, G. 1991. Effect of chlorophyllin on mercuric chloride-induced clastogenicity in mice. *Food Chem. Toxic.* **29**: 777–779.
- Johnston, P. J., MacPhail, S. H., Stamato, T. D., Kirchgessner, C. U. and Olive, P. L. 1998. Higher-order chromatin structure-dependent repair of DNA double-strand breaks: Involvement of the V(D)J recombination double-strand break repair pathway. *Radiat. Res.* **149**: 455–462.
- Mitscher, L. A., Telikepalli, H., Wang, P. B.-B., Kuo, S., Shankel, D. M. and Stewart, G. 1992. Antimutagenicity of secondary metabolites from higher plants. *Mutat. Res.* **267**: 229–241.
- Negishi, T., Ray, H. and Hayatsu, H. 1997. Antigenotoxicity of natural chlorophylls. *Mutat. Res.* **376**: 97–100.
- Pereira, A. A. B. 1991. Testes Estatísticos para Comparar Proporções em Problemas de Citogenética. In: Rabello-Gay, M. N., Rodrigues, M. A. L. R. and Monteleone-Neto, R. (eds.). *Mutagênese, Carcinogênese e Teratogênese: Métodos e Critérios de Avaliação*. Revista Brasileira de Genética, Ribeirão Preto, SP, Brasil. pp. 113–121.
- Pimentel, E., Cruces, M. P. and Zimmering, S. 1999. On the persistence of the radioprotective effect of chlorophyllin (CHLN) in somatic cells of *Drosophyla*. *Mutat. Res.* **446**: 189–192.
- Preston, R. J., Au, W., Bender, M. A., Brewen, J. G., Carrano, A. V., Heddle, J. A., McFee, A. F., Wolf, S. and Wassom, J. S. 1981. Mammalian *in vivo* and *in vitro* cytogenetic assays: A report of de U. S. EPA's Gene-Tox Program. *Mutat. Res.* **87**: 143–188.
- Rampazo, L. G. L., Jordão, B. Q., Vicentini, V. E. P. and Mantovani, M. S. 2002. Chlorophyllin antimutagenesis mechanisms under different treatment conditions in the micronucleus assay in V79 cells. *Cytologia* **67**: 323–327.
- Renner, H. W. 1990. *In vivo* effects of single or combined dietary antimutagens on mutagen-induced chromosomal aberrations. *Mutat. Res.* **244**: 185–188.
- Romert, L., Curvall, M. and Jenssen, D. 1992. Chlorophyllin is both a positive and negative modifier of mutagenicity. *Mutagenesis* **7**: 349–355.
- Sarkar, D., Sharma, A. and Talukder, G. 1994. Chlorophyll and chlorophyllin as modifiers of genotoxic effects. *Mutat. Res.* **318**: 239–247.
- , — and — 1996. Chlorophyll and chromosome breakage. *Mutat. Res.* **360**: 187–191.
- Sen, S., Sharma, A. and Talukder, G. 1991. Inhibition of clastogenic effects of nicotine by chlorophyllin in mice bone marrow cells *in vivo*. *Phytother. Res.* **5**: 130–133.
- Stopper, H., Full, M., Helbig, R. and Speit, G. 1997. Micronucleus induction by neocarcinostatin and methylmethanesulfonate in ionizing radiation-sensitive Chinese hamster V79 cell mutants. *Mutat. Res.* **383**: 107–112.