Mitotic segregation induced by edulcorant l-aspartyl-l-phenylalanine-metyl-ester (aspartame) in diploid cells of *Aspergillus nidulans*

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ABSTRACT. This study aims to verify the recombinogenic effect of artificial edulcorant l-aspartil-l-phenylalanine-metyl-ester (aspartame), widely used in hypocaloric and low-glucose diets. Such effect may reflect cell response to genetic damage, induced by artificial edulcorant in the cell cycle G_2 period and shows its capacity to induce homozygosis of recessive genes, previously present in heterozygosis.

Key words: carcinogenesis, edulcorant, recombinogenesis, heterozygosis loss.

RESUMO. Segregação mitótica induzida pelo edulcorante L-Aspartil-L-Fenilalanina-Metil-Ester (aspartame) em células diplóides de *Aspergillus nidulans* O presente trabalho demonstra o efeito recombinagênico do edulcorante artificial L-Aspartil-L-Fenilalanina-Metil-Ester (aspartame), amplamente utilizado em dietas hipocalóricas e de baixo consumo de glicose. Este efeito pode refletir respostas celulares a danos genéticos induzidos pelo edulcorante artificial, no período G2 do ciclo celular e demonstra sua capacidade de induzir homozigose de genes recessivos previamente presentes em heterozigose.

Palavras-chave: carcinogênese, edulcorantes, recombinogênese, perda de heterozigose.

Introduction

Irreversible DNA changes, caused by chemical compounds or ionizing radiations, may start a carcinogenesis complex process (Peto *et al.,* 1975). Several genetic mutations, involving many types of proteins, take part in the transformation process of a normal cell into a neoplasmic one (Duesberg *et al.,* 1999; Minamoto *et al.,* 1999).

Epidemiological and experimental studies show that several factors may cause important alterations in the carcinogenesis cell process. Pesticides and fungicides (Brownson *et al.,* 1990; Baptista and Castro Prado, 1997), medicinal compounds (Holliday, 1964; Demopoulos *et al.,* 1982; Franzoni *et al.,* 1997) and industrial chemicals (Thomas *et al.,* 1986; Hagmar *et al.,* 1990) are worth mentioning.

Whereas cancer is a progressive phenomenon which develops from a critically mutation-bearer cell (initiated cell), cell proliferation (tumor promotion) is necessary for the fixation of the first mutation. On the other hand, cell replication increases the probability of the occurrence of other mutations in decisive genes (tumor progression) (Nowel, 1976). Mutations in proto-oncogene and tumor suppressor genes constitute important changes for the development of neoplasm (Vogelstein *et al.,* 1989; Boyd and Barret, 1990). Although normal cells may be mutation bearers in tumor suppressor genes, their heterozygosis loss, caused by aberrant mitotic segregation, may be a promoting factor of neoplasm (Zimmermann *et al.,* 1966; Kinsella and Radman, 1978).

Mitotic crossing-over represents an important function in expression of recessive mutations in heterozygous diploid cells. Homologous chromatids may establish contact among themselves during mitosis and trigger the mitotic exchange process. Segregation of a recombinant and a parent chromatid towards the same mitotic pole will result in distal gene homozygosis at the point of exchange (Roper and Pritchard, 1955; Beumer *et al.,* 1998). Recessive genes homozygosis, induced by mitotic crossing-over, may thus promote a neoplasm event in heterozygous cells, in the case of mutations in tumor suppressor genes (Faruqi *et al.,* 1994; Laird and Jaenisch, 1996).

Agents recognized as neoplasm inducers are those that trigger the carcinogenesis process in initiating cells, even though incapable of separately inducing mutagenesis or carcinogenesis (Trosko *et al.,* 1977; Lankas *et al.,* 1977). The evaluation of recombinogenic potentiality of chemical compounds is of paramount importance since it detects those compounds that may be promoters in the carcinogenesis process (Chichetta and Castro-Prado, 2002*a*,*b*).

Artificial edulcorant aspartame (L-aspartyl-L-phenylalanine-metyl-ester) is widely used in hypocaloric diets, with a sweetening potential 200 times higher than saccharose. Edulcorant is formed by the combination of two amino acids, namely, aspartic acid and phenylalanine, and a metyl-ester group. Both amino acids occur naturally in almost all proteins, whereas methanol (metyl-ester group) is widely distributed among polysaccharids in fruits and vegetables (Lipton *et al.,* 1991).

Since it is vital to analyze the influence of environmental factors in human life quality and to evaluate the recombinogenic potential of the above, current research focuses on the genotoxicity of aspartame in heterozygous diploid cells of *Aspergillus nidulans*.

Material and methods

Strains and culture media

Strains UT196 and UT448 (Table 1) formed diploid UT448/UT196. Culture media were Minimum Medium (MM) (Kafer, 1977); Czapek-Dox prepared with 1% glucose; Complete Medium (CM) with yeast extract, casaminoacids, nucleic acids and vitamin solution (Kafer, 1977). Solid medium was prepared with 1.5% agar. Incubation occurred at 37°C.

Table 1. Genotype and origin of strains

Strain	Genotype	Origin
UT196	*yA2* (I), *methA17* (II), *pyroA4* (IV)	Utrecht (Holland)
UT448	*wA2* (II), *riboA1*(I), *pabaA124* (I), *biA1*(I), *AcrA1*(II)	Utrecht (Holland)

riboA1, *pabaA124*, *biA1*, *methA17*, *piroA4*: requirements for riboflavin, *p*-aminobenzoic acid, biotin, methionine and pyridoxine, respectively; *y* and w, yellow and white conidia, respectively; *AcrA1*, resistance to Acriflavine.

Techniques of genetic analysis

Diploid strains were obtained from heterokaryons prepared in MM + 2% CM, with suppression of agar (Roper, 1952). Aspartame toxicity was evaluated through diploid growth in edulcorant: diploid UT448/UT196 conidia were inoculated at the center of petri dishes with CM (control) and CM+aspartame (0.1 – 12.0 mg/ml). Six plates were inoculated for each aspartame dose and for control, and incubated at 37°C. Diameters of colonies were measured once a day, during five days, by a ruler. Means were compared by Student's *t*-test ($p<0.05$).

The recombinogenic capacity of aspartame was evaluated according to Franzoni *et al.* (1997). Aspartame (Sigma) was added to sterile CM at 45°C. Conidia of diploid UT448/UT196 were inoculated in petri plates with CM+aspartame and incubated for six days.

Each treatment produced visible sectors in diploid colonies, identified by their morphology and conidia coloration, which were different from those of the original diploid. The sectors formed were diploid, aneuploid or haploid mitotic segregants. After being purified in CM, segregants underwent a spontaneous haploidization process in CM. Only segregants that grew stably were chosen and labeled as haploids. Conidia of haploid segregants were individually transferred to 25 defined positions (5 x 5) in petri plates, with CM, for mitotic crossing-over analysis. Segregants were later transferred to more adequate selective media for phenotypic analysis.

Frequency of mitotic recombination between two markers in the same chromosome was taken as a percentage (FR = total number of recombinants x 100 ÷ total number of mitotic segregants). Results were statistically analyzed by Contingency Table 2 x 2 (Yates corrected λ^2) for $p<0.05$.

Results

Diploid UT448/UT196 growth was analyzed in CM with aspartame in concentrations ranging between 0.1 and 12.0 mg/mL. Change in growth rate occurred only in 12.0 mg/mL aspartame dose (Figures 1-2).

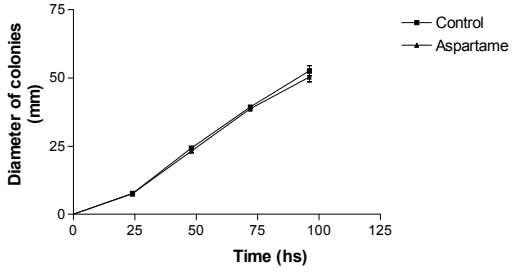

Figure 1. Growth of diploid UT448//UT196 in medium with aspartame (1.0 mg/ml)

Since aspartame doses 0.8 and 1.0 mg/ml induced higher frequencies of mitotic sectors per colony, they were chosen for the recombinogenesis tests. In doses 6.0, 8.0, 10.0 and 12.0 mg/ml aspartame induced intense changes in the colonies morphology (data not

shown), impairing the mitotic sectors isolating. In fact, only a few mitotic sectors have been observed in the cultivated colonies of the above-mentioned aspartame concentrations (Table 2).

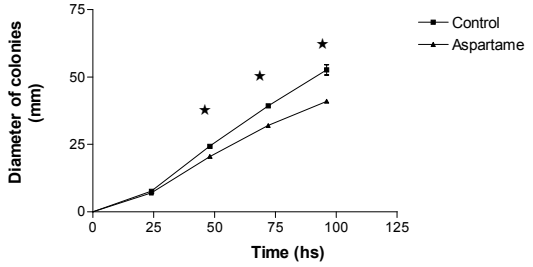

Figure 2. Growth rate of diploid UT448//UT196 with aspartame (12mg/ml). (*), significantly different from control, p< 0,05 (Student's t-test)

Table 2. Number of haploid mitotic segregants obtained from UT448//UT196 after aspartame treatment

Aspartame (mg/mL)	Average number of segregants/100 colonies
Control	43
0.1	43
0.2	45
0.6	48
0.8	87*
1.0	80*
6.0	20*
10.0	3*
12.0	3*

(*), significantly different from control, p< 0,05 (Yates corrected Chi-Square)

Mitotic crossing-over has been studied by means of phenotypes of haploid mitotic segregants from diploid UT448/UT196 with aspartame. Results showed aspartame's recombinogenic effect in both concentrations: 0,8 and 1,0 mg/mL (Table 3).

Discussion

The evaluation of aspartame genotoxicity is important owing to its high use in hypocaloric and low glucose consumption diets. Results show that aspartame at doses 0.8 and 1.0 mg/mL induces somatic segregation in diploid cells of *A. nidulans*.

Table 3. Frequencies of mitotic exchanges between genetic markers of chromosomes I and II, of mitotic haploids from diploid UT448//UT196 treated with 0.8 and 1.0 mg/mL of aspartame

Link interval	Frequencies of Mitotic Recombination (%)		
	Control	Treatment	
		A	B
y - bi	Zero (0/57)	15.2 (5/33)*	30.0 (9/30)*
Paba - bi	Zero (0/57)	10.1 (7/69)*	13.6 (9/66)*
Acr - w	Zero (0/170)	2.9 (2/69)	9.1 (6/66)*
Acr - meth	0.59 (1/170)	5.8 (4/69)*	9.1 (6/66)*
w - meth	0.59 (1/170)	5.8 (4/69)*	4.5 (3/66)

(A), treatment of diploid UT448//UT196 with aspartame 0.8 mg/ml; (B), treatment of UT448//UT196 strain with aspartame 1.0 mg/ml; (*) significantly different from control, p< 0,05 (Yates corrected Chi-Square)

Mitotic segregation in diploid strains of *A. nidulans*, heterozygous for conidia coloration genes (*y+/y* and *w+/w*), may be monitored by sectors that express *y* and *w* recessive alleles. These may be easily identified because of their conidia coloration (yellow and white), which are different from the original diploid with green conidia.

Participation of mitotic crossing-over in the recessive genes homozygotization process explains its involvement in carcinogenesis and teratogenesis processes. It seems that mitotic recombination starts from special recombinogenic sites that are subjected to the genotoxic effect of chemical substances (Holliday, 1964; Laird and Jaenisch, 1996; Baptista and Castro-Prado, 1997; Franzoni and Castro-Prado, 2000).

Changes in the morphology of diploid UT448/UT196 colonies occurred with aspartame doses over 6.0 mg/ml (data not shown). This fact shows their toxicity. On the other hand, aspartame doses between 0.1 and 1.0 mg/mL allow normal growth rates, without any changes in the colonies morphology. Doses between 0.8 and 1.0 mg/mL induced greater frequency in the mitotic segregants from diploid UT448/UT196 (Table 2).

Mitotic crossing-over has been analyzed in several gene intervals of chromosomes I and II and high frequencies of mitotic recombination were observed after the treatment of strain UT448/UT196 with 0.8 and 1.0 mg/mL aspartame (Table 3).

Amino acids in aspartame separate themselves after edulcorant ingestion. Metyl-ester grouping forms methanol, which is quickly converted into formaldehyde. The latter is known for its permanent genetic damages, such as chromosome aberrations, exchanges between sister chromatids and other severe damages in the neurological and immunological systems (O'Donovan and Mee, 1993; Trocho *et al.,* 1998; Spelt *et al.,* 2000).

Although aspartame's mutagenic effect has been observed only after *in vitro* nitrosation (Shephard *et al.,* 1993), current results show that the edulcorant may cause somatic recombination in diploid cells of *A. nidulans*. Since this effect may be the cell's response to genetic damage induced by the edulcorant, the latter may be characterized as an agent that causes homozygosis of deleterious genes previously present in cell genome. Results also suggest the participation of the compound and/or of its sub-products in basic cell processes, such as chromosome segregation and cell cycle.

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