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ORIGINAL ARTICLE

Chlorhexidine digluconate induces mitotic recombination in diploid cells of *Aspergillus nidulans*

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Chlorhexidine digluconate (1,1′-hexamethylene-bis[(5-*p*-clorophenyl)-biguanide]) is a bisbiguanidine antiseptic, used to decrease plaque formation and to control periodontal diseases. The determination of the frequency of mitotic crossing-over constitutes a very important method for detecting carcinogenic agents.

OBJECTIVE: The recombinogenic potential of chlorhexidine digluconate was evaluated on *Aspergillus nidulans* by the production of cells homozygous for the following nutritional markers: *riboA1*, *pabaA124*, *biA1*, *methA17* and *pyroA4*.

METHOD: *A. nidulans* was exposed to three concentrations of chlorhexidine digluconate (1, 5, and 10 μM).

RESULTS: Inhibition of colony development, conidiophore morphological alteration (cytotoxic effect), and the recombinogenic effect, indicated by homozygotization index (HI) values higher than 2.0, were observed for all concentrations of chlorhexidine digluconate. A homozygous *pyro+//pyro+* diploid strain and a diploid homozygous for the recessive *w* gene were isolated from UT448//A757 diploid treated with chlorhexidine digluconate, emphasazing its recombinogenic potential.

CONCLUSION: Although, beneficial effects of chlorhexidine, as an antiseptic agent, are reported in the literature, our results revealed that chlorhexidine digluconate, at less levels lowered those used clinically, caused toxic and recombinogenic effects on diploid *A. nidulans* strain.
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Keywords: mitotic crossing-over; loss of heterozygosity; oral antiseptic; Homozygotization Index; chlorhexidine

Introduction

Chlorhexidine digluconate {1,1′-hexamethylene-bis[(5-*p*-clorophenyl)-biguanide]} (CLX; Figure 1) is a bisbiguanidine antiseptic, widely used for topical preoperative skin disinfection and for treatment of skin wounds and as a surgical hand scrub (Mitchell *et al*, 1998). In dentistry, it has been utilized to decrease plaque formation, to reduce the collateral effects of radiotherapy and to control the development of periodontal diseases and caries (Foote *et al*, 1994; Hidalgo and Dominguez, 2001).

Chlorhexidine digluconate acts against Gram negative and positive bacteria, facultative anaerobes and aerobes, moulds, yeasts and viruses (Hidalgo and Dominguez, 2001).

In *Escherichia coli* it adversely affected the plasma membrane. Due to its chemical structure, CLX has a positive charge at physiological pH, resulting in non-specific interactions with phospholipids of the plasma membrane and thereby interfering with the cell's osmotic equilibrium. Furthermore, it induces rupture of the plasma membrane, leading to a rapid and irreversible loss of cytoplasmic components in a dose and time-dependent manner (Fitzgerald *et al*, 1992; Babich *et al*, 1995).

Cell proliferation is also affected by CLX. Hidalgo and Dominguez (2001), by incorporation of BrdU (5-bromo-2-deoxyuridine) into the DNA of proliferating cells, evaluated the effects of CLX on DNA synthesis. Chlorhexidine digluconate was cytotoxic to human dermal fibroblasts and, impeded these cells of advancing to S-phase of the cell cycle, and suppressed DNA synthesis.

In the LS5178Y mouse lymphoma (TK +/−) system, heterozygous at the thymidine kinase (TK) locus, Withrow *et al* (1989) evaluated the toxic and mutagenic potential of four chemical antiseptics used in ophthalmic solutions: benzalkonium chloride, chlorhexidine, thimerosal and ethylenediaminetetraacetic acid. Although chlorhexidine alone had little effect on the mutation rate, when the cells treated with CLX were exposed also to UVA radiation, there was an increase in the number of mutant cells.

Physical and chemical agents may induce chromosomal exchanges, and such an exchange acts as a promotional factor related to cancer development. Thus, the formation of neoplasms may be related to events of

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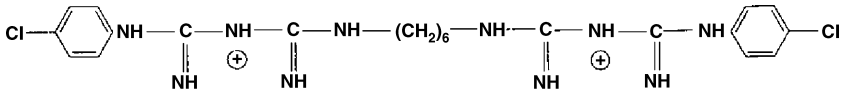

Figure 1 Chemical structure of chlorhexidine digluconate

somatic recombination by inducing homozygosity of those genes that control the cell cycle (Ringer and Schniper, 2000).

The filamentous fungus *Aspergillus nidulans*, constitutes an excellent organism to evaluate the frequency of mitotic crossing-over. This ascomycete spends a long period in the G2 phase of the cell cycle (Bergen and Morris, 1983), after chromosomes have duplicated, facilitating the occurrence of exchanges between homologous chromatids (Osman *et al*, 1993).

Considering that chlorhexidine has been widely used in dentistry specialties and studies have demonstrated the toxic effects of this compound on prokaryotic and eukaryotic cells, the present research evaluated the recombinogenic potential of this antiseptic towards diploid cells of *A. nidulans*.

Material and methods

Drug solution and strains

Chlorhexidine digluconate (20% aqueous solution) was obtained from Sigma Chemical Co (St Louis, MO, USA). The *A. nidulans* strains used are described in Table 1.

Culture media

Czapek-Dox with 1.0% (w/v) of glucose was used as the minimum medium (MM). The complete medium (CM) was previously described (Pontecorvo *et al*, 1953; Van de Vate and Jansen, 1978). Supplemented medium (SM) consisted of MM plus the nutriments required by each strain. Solid medium was amended with 1.5% agar. Incubation was at 37°C. General methodology followed previous reports (Roper, 1952; Pontecorvo *et al*, 1953). The diploid cells were prepared by the method of Roper (1952).

Drug cytotoxicity evaluation

Chlorhexidine digluconate was added to MM so achieve final concentrations of 1, 5 and 10 μM. Conidia of the diploid strain UT448//A757 were inoculated in plates containing MM (control) and MM + CLX (treatment). Five plates were used for each concentration, including the control. Diameters of colonies were measured after 24, 48, 72, 96 and 120 h of incubation. Colony diameters were compared by ANOVA, Bonferroni *post hoc* test, $P < 0.05$.

Recombinogenic test

UT448//A757 diploid strain conidia were inoculated in plates with MM (control) + CLX at 1, 5 and 10 μM. Plates were incubated for 6 days at 37°C. Visible diploid sectors (D1–D12), identified by differentiated morphology of the original diploid, were produced. Haploid mitotic segregants from diploid D1 to D12, obtained by spontaneous haploidization, were purified in CM. Only mitotic stable segregants were selected for the recombinogenic test and determination of the Homozygotization Indexes (HI) (Pires and Zucchi, 1994; Franzoni *et al*, 1997).

Determination of Homozygotization Indexes

The HI is based on the rate of formation of prototrophic and auxotrophic segregants. The proportion prototrophic/auxotrophic segregants would be 4+/4− if CLX did not induce mitotic crossing-over and would be 4+/2− if recombinogenesis occurred (Figure 2). Thus, HI values equal to or over to 2.0 indicate that CLX induced recombinogenesis (Pires and Zucchi, 1994; Chiuchetta and Castro-Prado, 2002a). HI was determined for each nutritional marker after the phenotypic analyses of the haploid mitotic segregants. Results were compared by Yates' continuity corrected chi-square test, $P < 0.05$.

Cytological analysis

Chlorhexidine digluconate was added to MM plates to achieve final concentrations of 1, 5 and 10 μM. Conidia of the diploid strain were inoculated and cultivated over dialysis membranes supported by MM + CLX. Plates were incubated at 37°C and samples were collected, stained with lactophenol cotton blue (Dring, 1971), and examined under a light microscope after 24 and 48 h of exposure.

Table 1 Genotype of strains

Strains	*Genotype*	*Origin*
UT448	*wA2*, *riboA1*, *pabaA124*, *biA1* (I), *AcrA1*	Utrecht
A757	*yA2*, *methA17*, *pyroA4*	FGSC

Mutant alleles give the following phenotypes: *ribo*, *paba*, *bi*, *meth*, *pyro*, requirements for riboflavin, *p*-aminobenzoic acid, biotin, methionine and pyridoxine respectively; *y* and *w*: yellow and white colouring of conidia, respectively; *Acr*, resistant to Acriflavine. FGCS: Fungal Genetic Stock Center (University of Kansas Medical Center, Kansas, USA).

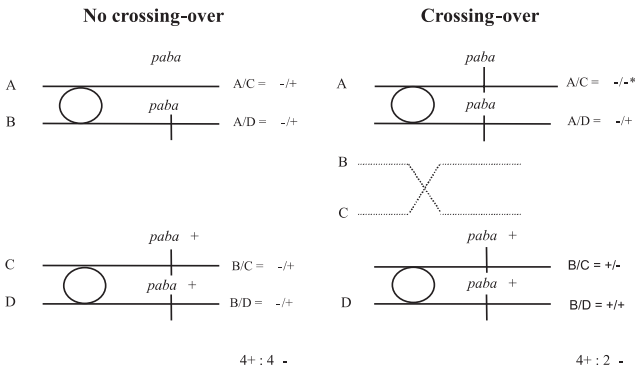

Figure 2 Origin of heterozygous and homozygous diploid segregants through mitotic crossing-over. *Not grown in MM

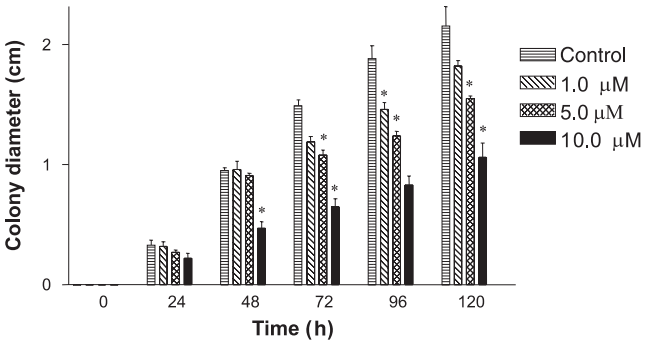

Figure 3 Growth of UT448//A757 diploid strain in minimum medium + chlorhexidine digluconate in 1.0, 5.0 and 10.0 μM concentrations. *Significantly different from control, ANOVA, Bonferroni hoc *post test*, $P < 0.05$

Results

Chlorhexidine digluconate, at concentrations of 1–10 μM (which are lower than those clinically used), reduced fungus growth. Initial reduction in colony growth was evident after 48 h with 10 μM (Figure 3).

A minimum of 171 mitotic segregants were isolated and analyzed for recombination induced by CLX. The recombinogenic potential of CLX was evaluated by the determination of HIs for *riboA1*, *pabaA124*, *biA1*, *methA17* and *pyroA4* nutritional markers. HI values higher than 2.0 were obtained in three tested concentrations of CLX (Table 2).

The recombinogenic effect of CLX was also recognized by the production of a homozygous *pyro+//pyro+* diploid strain (D6) at 1 μM CLX (Table 2). In addition, although auxotrophc diploids (−/−) are not selected in MM + CLX, a diploid homozygous for the recessive *w* gene (conidia coloration marker) was isolated from the UT448//A757 diploid, treated with 10 μM CLX (results not shown). Phenotypic analysis of both *pyro+//pyro+* (D6) and *w//w* diploids showed they were recombinant for *centromere-pyro* and *centromere-w* intervals, respectively.

Cytological analysis demonstrated that CLX produced morphological alterations in the UT448//A757 diploid strain of *A. nidulans*. Abnormal conidiophores, with bifurcations and malformed vesicles containing abnormal metulae and phialides were observed at three tested CLX concentrations (Figure 4).

Discussion

The toxicity of CLX towards *A. nidulans* might reflect damage to the plasma membrane. The reduction of fungal development observed at all CLX concentrations, as well as the formation of aberrant conidiophores, emphasizes the toxic effects of this antiseptic on *A. nidulans* cells.

Elevated HI values at the CLX concentrations were observed (Table 2). Diploid 6, a prototrophic recombinant diploid, homozygous for the *pyro+* gene, was produced when the UT448//A757 diploid strain was treated with CLX at 1 μM. Thus, as the number of *pyro* segregants was zero, the diploid 6 strain was not included in HI calculations for this genetic marker. Although the HI value was lower than 2.0 (HI = 1.5), the occurence of the *pyro+//pyro+* recombinant diploid demonstrated the recombinogenic effect of CLX on heterozygous diploid cells of *A. nidulans*.

The increase in the rate of mitotic crossing-over was a response to CLX. In spite of the high cytotoxic effects at 10 μM CLX, the recombinogenic effect was still noted (Table 2).

Tumour suppressor genes have been reported to confer negative regulatory controls on neoplastic cell transformation, which may be lost due to chromosomal exchange during malignant tumor development (Wong *et al*, 1996). The loss of heterozygosity in tumour suppressor gene was observed in oral cancer tissues (Kannan *et al*, 2001). Studies from Western countries have reported a high frequency of the loss of heterozygosity in *p53*, *retinoblastoma* (*rb*) and *adenomatous polyposis coli* (*apc*) genes in oral cancer tissues (Munirajan *et al*, 1996).

Table 2 Homozygotization Indexes (HI) of nutritional markers from diploid strain UT448//A757 exposed to chlorhexidine digluconate in 1.0, 0 and 10.0 μM concentrations

	Number of segregants																			
	Control					*1.0 μM*					*5.0 μM*					*10.0 μM*				
Genetic markers	*D1*	*D2*	*D3*	*T*	*HI*	*D4*	*D5*	*D6*	*T*	*HI*	*D7*	*D8*	*D9*	*T*	*HI*	*D10*	*D11*	*D12*	*T*	*HI*
ribo+	45	43	53	141	1.6	48	48	38	134	2.7*	38	50	61	149	6.8*	50	52	33	135	3.8*
ribo	29	25	34	88		19	15	16	50		12	06	04	22		18	7	11	36	
paba+	45	43	52	140	1.6	48	49	38	135	2.7*	37	50	61	148	6.4*	50	52	34	136	3.8*
paba	29	25	35	89		19	14	16	49		13	06	04	23		18	7	10	35	
bi+	45	42	49	136	1.5	37	46	39	122	1.9	38	50	61	149	6.8*	52	50	34	136	3.8*
bi	29	26	38	93		30	17	15	62		12	06	04	22		16	9	10	35	
meth+	48	44	57	149	1.9	57	47	34	138	3.0*	32	52	62	146	5.8*	50	38	42	130	3.2*
meth	26	24	30	80		10	16	20	46		18	04	03	25		18	20	2	40	
pyro+	32	34	53	119	1.1	40	38	54[a]	78	1.5	47	60	24	131	3.3*	43	43	38	124	2.7*
pyro	42	34	34	110		27	25	0	52		09	05	26	40		26	26	6	46	

[a]Not included in HI calculus; *Significantly different from control Yates' continuity corrected chi-square test.

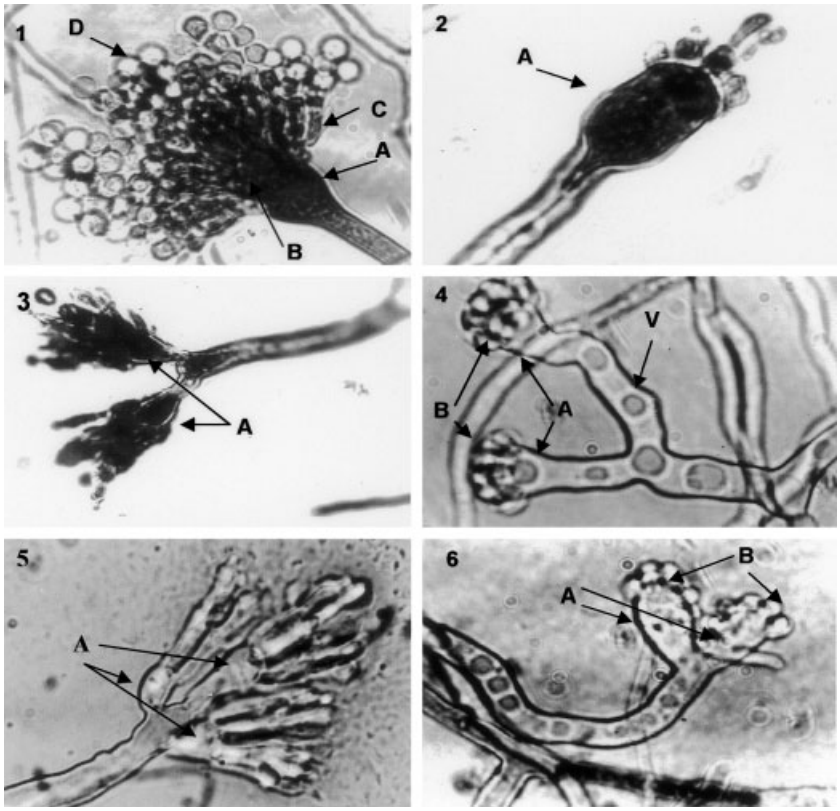

Figure 4 Cytological analysis of UT448//A757 diploid strain cultivated in the presence of chlorhexidine digluconate. (1)[a] Control (UT448//A757 normal conidiophore). (2)[b] Malformed conidiophore vesicle (1.0 μM). (3)[a], (5)[a] and (6)[b] Conidiophores with secondary vesicles (5.0, 10.0 and 10.0 μM, respectively). (4)[b] Malformed conidiophore with a secondary conidiophore (5.0 μM). **A**, vesicle; **B**, metulae; **C**, phialide; **D**, conidia; **V**, vacuole. [a]Incubated for 30 h; [b]incubated for 48 h. Vesicle diameter: 10 μm

Mitotic recombination leads the expression of recessive genes in heterozygous cells. Since tumor suppressor genes may be completely suppressed in recombinant cells, studies have been performed to identify the recombinogenic potential of several chemical agents (Chiuchetta and Castro-Prado, 2002b; Souza-Júnior *et al*, 2004). Thus, the determination of mitotic crossing-over frequency induced by chemical substances constitutes a very important method for studying carcinogenic agents (Wang *et al*, 1988).

The recombinogenic effect of CLX may be related to the interference of this antiseptic on DNA synthesis (Hidalgo and Dominguez, 2001). Agents able to inhibit DNA synthesis also interfere in the frequency of mitotic exchanges (Esposito and Holliday, 1964; Holliday, 1964; Franzoni and Castro-Prado, 2000).

Although other research has demonstrated the beneficial potential of chlorhexidine as an antiseptic, our results reveal that CLX, at levels lower than used clinically, was cytotoxic towards *A. nidulans* and induced DNA recombination leading the occurrence of gene homozygosity. Further work has to be performed to conclusively demonstrate recombination events triggered by CHX.

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