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Chlorhexidine induces DNA damage in rat peripheral leukocytes and oral mucosal cells

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Objective: Chlorhexidine digluconate is widely used in dental practice for decreasing plaque control, controlling gingivitis and disinfecting root canals. However, the undesirable effects of chlorhexidine digluconate regarding its genotoxicity are conflicting in the literature. Thus, the aim of this study was to investigate the genotoxicity of chlorhexidine digluconate in rat peripheral blood and oral mucosal cells by the single cell gel (comet) assay and micronucleus assay.

Methods: Thirty male Wistar rats were distributed into three groups: negative control; experimental group orally treated with 0.5 ml of 0.12% chlorhexidine digluconate, twice daily, during 8 days; and positive control, which received 4-nitroquinoline 1-oxide at 0.5 g/l by drinking water.

Results: A statistically significant increase of DNA damage was observed in leukocytes and oral mucosal cells of the chlorhexidine digluconate treated group, as assessed by the comet assay. However, no increase of micronucleated cells was detected in reticulocytes from peripheral blood cells.

Conclusions: Taken together, the data indicate that chlorhexidine digluconate is able to induce primary DNA damage in leukocytes and in oral mucosal cells, but no chromosome breakage or loss in erythrocytes.

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Chlorhexidine digluconate is a symmetrical cationic molecule containing two 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain (1). Although it is widely used in dental practice (2) for decreasing plaque formation, gingivitis controlling and disinfecting root canal (3, 4), the data about its genotoxic potential are still conflicting. Increased DNA damage in buccal cells was detected by the single cell gel (comet) assay in individuals that rinsed their mouths with chlorhexidine digluconate (1). Genetic mutations were also induced by the breakdown products of chlorhexidine digluconate in microorganisms (5). Nevertheless, negative results were detected in the SOS chromotest and UMU test (6, 7).

It is becoming increasingly evident that an increased rate of DNA damage and chromosome breakage or loss is an important risk factor for elevated risk for cancer (8, 9). A variety of *in vitro* and *in vivo* test systems is available for evaluating early genetic damage induced by xenobiotics, being the peripheral blood micronucleus assay in rodents widely used for detection of cytogenetic damage (10). The micronucleus is defined as microscopically visible, round or oval cytoplasmic chromatin masses next to the main nucleus, arisen from chromosomal fragments or whole chromosome not incorporated into the daughter nuclei during cell division (10, 11). Compared to similar assays (chromosomal aberrations and sister chromatid exchanges), the micronucleus test lies easier for screening of chromosomal defects in cytological specimens. The single cell gel (comet) assay is a rapid, simple, sensitive and reliable biochemical technique for evaluating DNA damage in cells or in tissues from which viable cell preparations can be obtained (12). In this assay, a small number of cells are embedded in an

agarose gel, lysed by detergents, electrophoresed for a short time under alkaline conditions (pH > 13) and stained with a fluorescent DNA binding dye. Cells with increased DNA damage (especially single-strand breaks and alkaline-labile sites) display increased migration of the DNA from the nucleus towards the anode resembling a comet image. Thus, the amount of DNA migration indicates the amount of DNA breakage in the cell (13). Nowadays, the comet assay has been used as an important approach for the assessment of genetic damage at low level of exposure (13, 14).

Considering the conflicting *in vivo* evidence about the genotoxicity of chlorhexidine digluconate, the current study was designed to evaluate, under controlled experimental conditions, whether this antiseptic compound can cause DNA damage in rat oral mucosa and peripheral blood cells. The comet assay was performed in leukocytes and oral mucosal cells and the micronucleus test in peripheral erythrocytes of Wistar rats.

Materials and methods

Animals and experimental design

All experimental protocols used in the present study were approved by the Ethical Committee for Animal Research, UNESP, Botucatu, Brazil.

Eight-week-old male Wistar rats, weighing approximately 250 g, were obtained from Centro de Bioterismo, UNICAMP (CEMIB), Campinas, SP, Brazil, and maintained under controlled conditions of temperature (22 ± 2°C), humidity (50 ± 10%), and 12 h light/dark cycle, with free access to commercial diet (Nuvilab CR1 from Nuvital, Curitiba, Brazil) and filtered water. The animals were randomly distributed into three groups: Group I, negative control; Group II, orally treated with 0.5 ml of 0.12% chlorhexidine digluconate (Periogard®, Colgate-Palmolive Co., Sao Paulo/SP, Brazil), twice daily, during 8 days, through disposable syringe; Group III (positive control), treated intraperitoneally with 4-nitroquinoline 1-oxide (Sigma, St Louis, MO, USA) dissolved in tap water at final concentration of 0.05 g/l. All the animals were killed by pentobarbital intraperitoneal injection (40 mg/kg, body wt) at the end of the experimental period (8 days).

After death, a volume of 1 ml of peripheral blood was collected from the heart using a fine needle. Oral mucosal cells were collected from the hard palate, the cheek mucosa and the floor of mouth, using a wood spatula. Cells were placed into a tube containing 1 ml of cold phosphate buffer solution (Ca^{2+}, Mg^{2+} free) and centrifuged at 180 *g*, during 5 min, at room temperature (25°C). The supernatant was removed and the cell suspension was used in the comet assay.

Single cell gel (comet) assay

The alkaline comet assay was performed under undirected light, following the guidelines purposed by Tice *et al*. (15), with some modifications. Briefly, 10 µl of peripheral blood or oral cell suspension were added to 120 µl of 0.5% low-melting point agarose at 37°C, layered onto a precoated slide with a thin layer of 1.5% regular agarose, covered with a coverslip, and placed at 4°C for 5 min, in order to solidify the agarose. The coverslip was removed and the slides immersed into a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl buffer, pH 10, 1% sodium sarcosinate, 1% Triton X-100, and 10% dimethylsulfoxide, added just before use) for approximately 1 h. Then, the slides were placed into a horizontal electrophoresis unit filled with a freshly made alkaline buffer (1 mM EDTA and 300 mM NaOH, pH 13). After a 20-min DNA unwinding period, electrophoresis was carried out at 25 V (0.86 V/cm) and 300 mA for 20 min. For oral mucosal cells, the slides were treated with proteinase K (Life Technologies, Carlsbad, CA, USA) (10 mg/ml, pH 8) for 1 h, at 37°C. Unwinding and electrophoresis times were conducted at both 20 and 10 min, respectively. After electrophoresis, the slides were neutralized in a buffer (0.4 M Tris at pH 7.5), dehydrated in absolute ethanol and dried at room temperature. Before analysis, the slides were stained with 50 µl ethidium bromide (20 µg/ml). Fifty randomly selected cells per animal were examined at 400 × magnification in a fluorescence microscope, using an automated image analysis system (Comet Assay II, Perceptive Instruments, Haverhill, UK). Two metric parameters were selected as indicators of DNA damage: tail intensity (% tail DNA, in percentage of pixels), and tail moment (product of tail DNA/total DNA by the tail center of gravity, in arbitrary units).

Micronucleus assay

Micronucleus assay in peripheral blood erythrocytes was performed according to a protocol previously described (10). Briefly, 5 µl of peripheral blood was taken from the rat ocular vein and stained with acridine orange (1 µg/ml). Frequencies of micronucleated cells were blindly recorded based on the observation of 1000 reticulocytes per animal in a fluorescence microscope at 1000 × magnification.

Statistical methods

The data from the comet and micronucleus assays were assessed by the Kruskal–Wallis non-parametric test and by the Chi-square test, respectively, using the software SigmaStat, version 1.0 (Systat Software, Inc., Point Richmond, CA, USA) for Windows. p-value < 0.05 was considered for statistic significance.

Results and discussion

Table 1 shows the data obtained in the comet assay. A statistically significant increase of DNA damage was detected in peripheral leukocytes and in oral mucosal cells of the animals exposed to chlorhexidine digluconate. Conversely, no increase of micronucleated cells was observed in reticulocytes, indicating that chlorhexidine digluconate did not induce chromosome breakage or loss in erythrocytes (Table 2).

The present study focused on DNA injury as a result of the effect of the

Table 1. DNA damage (tail moment and tail intensity) in peripheral leukocytes and oral mucosa cells of male Wistar rats orally exposed to chlorhexidine digluconate (CHD)

	DNA damage			
	Tail moment		Tail intensity	
Groups	Leukocytes	Oral mucosa	Leukocytes	Oral mucosa
Negative control	0.20 ± 0.07	0.38 ± 0.10	2.79 ± 0.48	3.47 ± 0.58
Chlorhexidine digluconate[a]	1.33 ± 0.54*	1.34 ± 0.46*	10.00 ± 2.90*	9.02 ± 1.95*
Positive control[b]	1.82 ± 0.54*	1.74 ± 0.40*	8.21 ± 2 .66*	8.77 ± 1.62*

[a]Chlorhexidine digluconate at 0.12%, twice a day, during 8 days.
[b]4-Nitroquinoline 1-oxide, 0.05 g/l, by drinking water.
*$p < 0.05$ (compared to the negative control).

Table 2. Frequencies of micronucleated reticulocytes in male Wistar rats orally exposed to chlorhexidine digluconate

		Micronucleated reticulocytes	
Groups	No. of cells analyzed	No.	%
Negative control	10000	14	0.14
Chlorhexidine digluconate[a]	10000	13	0.13
Positive control[b]	10000	41	0.41*

[a]Chlorhexidine digluconate at 0.12%, twice a day, during 8 days.
[b]4-Nitroquinoline 1-oxide, 0.05 g/l, by drinking water.
*$p < 0.05$ (compared to the negative control).

chlorhexidine digluconate on peripheral blood and oral mucosal cells *in vivo*. As oral mucosa is the first contact site to the chlorhexidine digluconate, and blood reflects the systemic conditions (16), cells from both tissues were chosen for evaluating DNA damage. *In vivo* protocols investigating the genotoxic potential of chlorhexidine digluconate are rare in the literature. On the other hand, age, gender, dietary habits and smoking status are confounding factors in human evaluation. Although dietary habits have been long associated with the high risk for mutation-related diseases such as cancer, it is very difficult to include or exclude their noxious activities in the biomonitoring studies of human population. Thus, the current study was conducted using an experimental system, which makes possible the evaluation of the effects under controlled conditions, especially regular diet.

The alkaline version of the comet assay is sensitive for a wide variety of DNA lesions (single-strand breaks, double-strand breaks, and alkali-labile sites). Recently, we have used this assay to assess the putative genotoxicity of several compounds used in dental practice (17, 18). The data reported here demonstrated that chlorhexidine digluconate intake induces primary DNA damage in rat leukocytes and oral mucosal cells. A similar result was previous described using the comet assay (1). However, a negative result was observed in the SOS chromotest and the UMU test (6, 7). A possible explanation for chlorhexidine digluconate-induced primary DNA damage is based on the hypothesis that chlorhexidine digluconate and its related compounds may bind to proteins, which contribute to alterations of cellular functions and induce DNA damage. However, it is still not clear how chlorhexidine digluconate is absorbed from the oral mucosa and gastrointestinal tract to promote these biological actions.

It is generally accepted that primary DNA damage may lead to chromosomal aberration and, subsequently, to the formation of micronuclei after one cell cycle division. This correlation may not be trivial, since the formation of chromosome breakage depends on the types of damage and on the ability of the cell to repair the damage (19). Thus, we also attempted to demonstrate whether the level of DNA damage detected in leukocytes by the comet assay was associated with the micronucleus frequency. Our results clearly showed no relationship between primary DNA damage and chromosomal breakage or loss. However, it must be noted that the primary DNA damage was detected in peripheral leukocytes, whereas the micronucleus was evaluated in erythrocytes. Perhaps, chlorhexidine digluconate was not able to reach the bone marrow during the erythroblastosis, when micronuclei are formed. Recently, chlorhexidine digluconate-induced chromosomal damage in the mouse lymphoma assay was described by some investigators (20, 21).

In conclusion, the present study indicates that chlorhexidine digluconate induces DNA damage in both rat peripheral and mucosa buccal cells, but not the micronucleus in erythrocytes. Besides the contribution to the evaluation of the potential health risk associated with compounds widely used in dental practice, our results highlight the need for a better understanding of the mechanism of chlorhexidine digluconate concerning its genotoxicity.

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