

Assessment Of Cytotoxicity Of Chlorhexidine Containing Mouthrinses By Micronucleus Test In Exfoliated Buccal Epithelial Cells

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Abstracts: Background & Objective: Chlorhexidine mouth rinses are utilized worldwide on regular basis in oral hygiene practice for plaque control. Studies have shown that chlorhexidine has toxic effects on a variety of eukaryotic cells. Micronuclei count in exfoliative cells is an economical and non invasive diagnostic method for evaluation of cytotoxic effects of many carcinogens/ co-carcinogens. The present study was conducted in order to evaluate frequency of micronuclei in buccal epithelial cells of patients using chlorhexidine containing mouthrinses. Methodology: Study included 50 subjects, of whom 30 having clinically suspected mild gingivitis, rinsed with chlorhexidine; 20 control subjects, rinsed with saline. Buccal epithelial cells were collected with a brush before and after one week of usage of chlorhexidine by patients and physiologic saline by controls. Cells subjected to Feulgen reaction and analysed by two independent observers for micronuclei counts. Results: Considerable increase in micronuclei count was noted in patients using chlorhexidine compared to controls. Conclusion: Present study suggests that chlorhexidine has cytotoxic effects and further studies involving the therapeutic use of different mouthrinses for a longer duration may provide justification for their usage in clinical practice. [Vinod Kumar MP NJIRM 2016; 7(3): 75 - 79]

Key Words: micro nucleus test, chlorhexidine, exfoliative cytology, cytotoxicity, Feulgen reaction

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Introduction: Mouth rinses are utilized worldwide on regular basis in oral hygiene practice for plaque control. Chlorhexidine containing mouth rinses have been effective in decreasing plaque formation and controlling both gingivitis and dental caries¹.

Chlorhexidine digluconate (CHX) is a bisbiguanide antiseptic active against gram-positive and gram-negative bacteria, facultative anaerobes and aerobes, moulds, yeasts and viruses. Its antibacterial activity arises from its positive charge at physiological pH, which produces nonspecific binding to the negatively-charged membrane phospholipids of bacteria; this causes an alteration in bacterial osmotic equilibrium, with potassium and phosphorus leakage. As the CHX concentration increases, cytoplasmic contents precipitate, triggering cell death. Several studies have shown that CHX has toxic effects on a variety of eukaryotic cells, with the presumed cytotoxicity mechanism being related to electrostatic surface².

These cytotoxic effects can be assessed by various bioassays like flow cytometry, COMET assay and micronucleus test. Among aforementioned techniques micronucleus test is least expensive, simple and reliable bioassay and also an effective indicator of chromosomal damage in exfoliated cells from sites like lung, bladder, nasal and oral cavity^{3,4}.

Micronuclei (MN) arise from acentric fragments or whole chromosomes that are not included in the main

nuclei of the daughter cells. The formation of micronuclei can be induced by substances that cause chromosome breakage (clastogens) and by agents that affect the spindle apparatus (aneugens)⁵. Micronuclei rates therefore indirectly reflect chromosome breakage or impairment of the mitotic apparatus. The quantitative detection of micronucleus is widely used for analysis of cytogenetic damage. It is commonly used as a diagnostic tool for assessing the cytogenetic damage induced by numerous potential carcinogens including tobacco, pesticides, smoking etc⁶.

Frequency of micronuclei appears to increase in carcinogen-exposed tissues long before any clinical symptoms are evident. A rise in MN in exfoliated buccal cells indicates an increased risk for cancer from various carcinogens like tobacco, smoke, ionizing radiation etc⁷. The key advantage of the MN assay is the relative ease of scoring, the limited cost and precision obtained from scoring large number of cells⁸. Compared to the determination of chromosomal aberrations and sister chromatid exchanges an advantage of the micronucleus test lies in the easier and clearly increased screening of chromosomal defects in cytological specimens⁹. Therefore, application of MN test can be considered as a sensitive tool for analysis of cytogenetic damage in human populations for various carcinogens and mutagens.

Glorification of the beneficial effects of chlorhexidine and its over the counter access availability in countries like India has led to its injudicious and rampant use as a mouthwash. As mouthrinses are administered directly to the oral mucosa or skin wound, CHX should provide low cytotoxicity and a high safety level, qualities which we considered worth studying in more depth. So, the aim of the present study was to evaluate the detrimental effects of CHX by micronuclei count using Feulgen reaction.

Material and Methods:

Participants: The study included 50 patients (42 males and 8 females) who visited Department of Oral and Maxillofacial Pathology, Microbiology and Forensic odontology, P.M.N.M Dental College and Hospital, Bagalkot, Karnataka. Mean age of patients was 21.94 years. The institutional ethical committee clearance was obtained before starting the study. After obtaining an informed consent, detailed case history and clinical examination of the patient was recorded which included subjects' individual characteristics such as gender, age, habits, and exposure to genotoxic agents such as alcohol consumption or smoking habits etc. It was important that they had no caries or dental restorations; because it is known that some dental materials increase the frequency of micronuclei. Therefore, the subjects were chosen carefully among whose DMFT (Decayed Missing Filled Teeth) scores were zero.

Study population were distributed into three groups:

- Group I (test group): 30 patients with clinically suspected mild gingivitis patients; rinsed with chlorhexidine
- Group II (negative controls): 20 subjects without any oral lesions; rinsed with saline.

Group 1 and Group 2 patients were requested to rinse their mouth with 5 ml of chlorhexidine and physiologic saline respectively, twice a day for a week. Rinsing procedure took approximately 1 min each time.

Chemicals: The buffer solution required for the study was prepared in the Department of Pharmacology, College of Pharmacy, Bagalkot.

Sample collection: The material for analysis was obtained after thoroughly rinsing the mouth with water, by gently scraping the buccal mucosa of both sides with a mini toothbrush. The buccal epithelial cells were obtained before and after one week of usage of

chlorhexidine by the patients and physiologic saline by the controls. Cells were collected in a conical tube containing 25 ml of buffer solution (0.1M EDTA, 0.01M Tris HCl, 0.02M NaCl) pH 7.0 and washed by centrifugation at 1000 rpm for 10 minutes. An adequate cell suspension was dropped onto clean slides and cell density was checked using a microscope. The slides were allowed to dry and then fixed in 100% cold methanol for 30 minutes. Slides were aged at 37^oc overnight. Slides were stained with Feulgen staining technique and examined under light microscope using 100x magnification and frequency of micronuclei in buccal cells was evaluated by scoring 2000 cells from each person for each sampling time (before and after exposure). The scoring was done according to the criteria established by Tolbert et al¹⁰.

Results: Statistical analysis - The MN count was 1.06 before CHX usage and after usage, it increased to 1.56. On comparing the micronuclei counts before and after usage of CHX and saline by respective groups, a statistically significant difference with a p value of 0.0331(p<0.05) was noted, whereas in case of saline it was not significant.

Table 1: Comparison of Micronuclei counts, before and after rinse in cases and control groups by Wilcoxon matched pair test.

Group	Exposure	T-value	Z-value	p-level
Group 1	Before vs After	15.0000	2.1315	0.0331*
Group 2	Before vs After	30.0000	0.2667	0.7897

*p<0.05

On comparing the micronuclei counts in both groups 'before' exposure (See Table 1) using Mann Whitney U test, shows no statistical difference was found.

Table 2: Comparison of MN count in both groups 'before' exposure

Before exposure	Group 1	%	Group 2	%	Total
Score 0	1	3.33	4	20.00	5
Score 1	2	66.67	1	5.00	3
Score 2	3	10.00	2	10.00	5
Total	3	100.00	2	100.00	5
U-value	255.0000				
Z-value	-0.8911				
P-value	0.3729				

Similarly no statistically significant difference was found in both the groups 'after exposure' also with p value being 0.0655.

On applying Wilcoxon matched pair test in order to compare the MN count 'before and after' exposure for group 2, there was no statistical significance in the micronuclei counts of negative controls before (MN=18) and after (MN=19) exposure to saline. But a statistically significant difference was noted in the micronuclei counts before (MN=32) and after (MN=47) exposure to chlorhexidine i.e. group 1 with a p value of 0.0331(p<0.05).

Discussion: Biomarkers have been used to assist in diagnosis and staging of disease, as well as to evaluate the risk assessment. To date, a variety of assays have been proposed as potential biomarkers in biomonitoring studies, including those that assess metaphase chromosomal aberrations, sister chromatid exchanges and host cell reactivation. However, these methods are typically laborious, time-consuming and require highly trained technicians to accurately read and interpret the slides. For this purpose, a great deal of enthusiasm was raised by the application of the micronucleus test to exfoliated cells. Micronucleated cell indices may reflect genomic instability, because they arise due to chromosomal damage¹¹. Their frequency appears to increase in tissues exposed to carcinogens, tobacco, ionizing radiation and cytotoxic substances, long before any clinical symptoms are evident^{12, 13, 14, 15}. Therefore, in our study the analysis of micronuclei counts in exfoliated oral mucosal cells has been employed to study the cytotoxic effects of chlorhexidine.

Chlorhexidine (CHX) is a bisbiguanide with antiseptic properties, largely used in dentistry, mainly for management of periodontal problems and in oral pre-operative procedures¹⁶. Most commonly used as a mouthrinse, which are adjuncts to mechanical oral hygiene¹¹. Many experiments have been performed in vitro in an attempt to elucidate the mechanisms of action of CHX and have demonstrated its cytotoxic potential by inhibition of protein synthesis, induction of apoptosis at low concentrations and necrosis at high concentrations, in addition to inhibition of DNA synthesis. The cytotoxic potential of CHX can also be related to the length of cell exposure and CHX concentration¹⁷. And it has been suggested that the direct application of CHX during regenerative therapy for the treatment of periodontal and peri-implant diseases could have serious toxic effects on gingival fibroblasts, endothelial cells and, especially, on

alveolar osteoblasts, thus negatively interfering with the early healing phase of these oral diseases¹⁸. Hence the present study with larger sample size is taken up to evaluate the micronuclei counts before and after exposure to chlorhexidine compare that with saline used controls.

Site of collection of cells in the present study was buccal epithelial cells because as it is evidenced in the previous study that, cell types that repair DNA damage efficiently are likely to show lower levels of residual damage than cells less proficient in DNA repair¹⁹. Buccal cells have been shown to have limited DNA repair capacity relative to peripheral blood lymphocytes, and therefore may more accurately reflect genomic instability events in epithelial tissues.

Micronucleated cell indices may reflect genomic instability¹⁶. The detection of an elevated frequency of MN in a given population indicates an increased risk of oral cancer. Micronucleus tests have been less frequently applied to buccal epithelial cells of patients in detecting the possible cytotoxic and/or mutagenic effects of some mouthrinses in this anatomical area /tissue. The results of our study demonstrated that the mean MN count after exposure to CHX containing mouthrinses was 1.56 which is more than that of before exposure (1.06). In a study by Carlin et al¹¹, MN count was found to be 0.27 and 1.8 before and after exposure to CHX containing mouthrinses. In another study by Erdemir et al² found very high MN count after exposure to CHX containing mouthrinses, mean was found to be 11.86 and in non CHX containing it was 7.14.

The observations of this study affirm the suggestion of Erdemir et al² that the amount of absorbed mouthrinse may have been sufficient to induce MN frequencies in buccal epithelial cells of patients even in the range of biological tolerance levels of these mouthrinses. Further it is to note that a wide variations in the MN frequency counts as evident in the previous studies may be attributed to DNA specific stains used, wherein MN formation in epithelial cells may be overestimated when non- DNA specific stains are utilized⁵. Standardized and informed investigative tools to estimate the MN count in different tissues with minimal variations may be employed, which is very well illustrated in the present study.

In the present study, the difference in MN counts of negative control group, before (0.9) and after (0.96) exposure to saline did not show significant statistical difference. This shows that saline does not induce any cytotoxic effects on buccal epithelial cells similar to the study conducted by Erdemir et al².

Regarding the host factors many previous studies showed higher frequency in females and a uniform increase in frequency with age in both genders. In our study we could not find any difference in genders and all study subjects were young people so we could not find any difference in age.

Conclusion: Based on the observations of this large cohort study it appears that the use of CHX for plaque control even in therapeutic doses may induce MN formation indicating cytotoxicity. MN count with DNA specific stains is an economical and helpful method to assess the genotoxic and cytotoxic effects of hazardous substances in vivo.

Further such studies involving the therapeutic use of different mouthrinses in longer duration may provide justification for their usage in clinical practice.

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