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Cytotoxic effect of chlorhexidine gluconate mouthwash – A micronuclei-assay

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Abstract
Chlorhexidine Gluconate is a broad-spectrum antimicrobial drug that is widely used as mouthwash. Several clinical studies have proved its safety as a mouthwash when used within pharmacological dose limit. However, severe cytotoxic effects on cultured cell line and even in animal trials also have been reported. Considering these findings, the rationality of the continuous use of Chlorhexidine Gluconate mouth wash for longer duration becomes questionable. To evaluate the genotoxicity of Chlorhexidine Gluconate for long term use within pharmacological dose limit by estimation of Micronucleus index in exfoliated human buccal mucosal cells using Acridine Orange under fluorescence microscope. In result no significant difference in the Micronuclei count in the whole population was observed before and after use of the 0.2% Chlorhexidine mouth wash. Micronucleus count was also unaltered in the male and female population before and after use.

Keywords: Acridine Orange, Buccal mucosa, Chlorhexidine Gluconate, Cytotoxicity, Micronucleus.

1. Introduction
Chlorhexidine Gluconate (CHX) a potent antiplaque mouthwash, is active against many oral pathogens including gram positive and negative bacteria, viruses, even fungus [1-3]. It effectively maintains the gingival and periodontal health [4]. This drug has been proved to be safe for longer period continuous use without any visible oral or systemic effect [5-8]. However prominent side effects like taste alteration, tooth discoloration, and mucosal discoloration cannot be ignored [9-14]. CHX shows cytotoxicity and even causes dysplasia in the oral mucosa [15-17]. Many reports have supported chromosomal break down [16, 18] but the effects were found to be transient. So there is a need to reevaluate the cytotoxic effect of the drug on oral mucosa. In this study Micronucleus frequency in buccal mucosal cells was used to evaluate the cytotoxicity of 0.2% CHX. Micronucleus frequencies in the exfoliated buccal mucosal cells are one of the most frequently applied biosurveillance tools for monitoring the genotoxic damage [19]. The present study aimed at evaluating the cytotoxicity of 0.2% CHX.

2. Materials and methods
This study was done in the Dental College, Regional Institute of Medical Sciences, Out Patient Department from July 2014 to November 2014 after getting approval from the Institutional Ethical Committee and obtaining written consents from all the subjects. 60 subjects (30 male and 30 female) within the age group of 18 to 24 years without having any visible oral mucosal lesion were included in the study. A detailed questionnaire was prepared to rule out other contributing factors that can alter the Micronucleus frequency such as diet, vitamin supplement, medical history within last six months, history of past illness, history of radiation therapy, any deleterious oral habits such as smoking or chewing tobacco, consuming alcohol, or any drug abuse. Subjects with positive history that can alter micronucleus count were excluded from the study. Baseline samples of exfoliated buccal mucosal cells before 0.2% CHX use were collected for Micronucleus evaluation. Then, subjects were instructed to rinse their mouth with 10ml of the solution for 30 seconds twice daily for a period of 9 weeks. At the end of 9th week exfoliated cells were collected from each subject. Subjects were also evaluated for any visible changes in the oral mucosa and subjective symptoms like burning sensation, taste alteration or any other discomfort.

2.1 Method of collection of exfoliated cells

The exfoliated cells were collected by scraping the oral mucosa with a wooden spatula in rolling motion [20]. Collected cells were shaken in a centrifuge tube containing the saline solution to release the cells, and then the tube was centrifuged at 1500 rpm for 15 minutes to wash the cells in a buffer solution at pH 7.0 for removing bacteria and cell debris, which may confound the scoring. Then, the cells were transferred to slides by careful droppings with pipette followed by fixation with 100% ethanol [21, 22]. A total of 16 slides were prepared for each subjects, 8 slides from sample obtained before CHX use and 8 slides from sample obtained after CHX use.

The slides were stained with 1 % Acridine Orange **(**Loba Chemie, India) staining solution [23] and immediately observed under a Fluorescent microscope (Kyowa, Japan) under 10X and 40 X magnifications (Figure 1). The Micronucleus count was done by a single observer in zigzag method out of 1000 intact epithelial cells [21].

2.2 Statistical analysis

The difference in micronuclei incidence between males and females before and after use was compared by Mann-Whitney U test. The difference in the pre and post use incidence of Micronuclei within males and females was compared by Wilcoxon Signed Rank Test. P-value of <0.05 was considered as statistically significant.

3. Results

Table 1: It displays the result of the whole population which did not show significantly altered Micronucleus count before and after use of CHX mouth wash.

Table 2: It shows the results of micronucleus count for male and female before and after CHX use and the count was not statistically significantly altered.

4. Discussion

The ultimate means of maintaining oral hygiene is controlling the dental plaque [24]. Use of mouthwashes is a very common practice for the general population and a fundamental step for maintaining oral health in elderly and the handicapped people [25-28]. Among all the mouthwashes available, CHX is considered to be the Gold standard [29]. It is a well tolerant drug and when swallowed accidentally shows minimal metabolic changes and has a safe fecal excretion [1]. Though the drug has been found to be safe for clinical uses but it is known to produce allergic reactions like pain, burning sensation, puriritis and even aphthous-like lesion on the gingiva [11]. CHX is reported to be highly cytotoxic to peripheral blood neutrophils due to its lytic properties [30]. It induces apoptosis at low concentrations and necrosis at high concentrations [31]. in addition to inhibition of DNA synthesis leading to cell death [32]. This drug was also found to alter the cellular redox balance, resulting in increasing levels of free radical generation and finally causing death of the cell [33]. Sanchez *et al* in a paper in 1998 have supported the cytotoxic nature of the drug on canine embryonic fibroblastic cells and also commented that it is lethal to the fibroblast in bactericidal dose [34]. However, Ribeiro *et al* in a paper in 2005 reported that CHX at the concentration ranging from 0.1% -1% is not cytotoxic to Chinese hamster ovary (CHO) cells [35]. On the contrary Li YC *et al* reported in 2014 in their *in vitro* study, that the cytotoxicity of CHX on macrophages is concentration dependent and the drug induces its cytotoxicity via ROS generation [36].

The cytotoxic effect of CHX *in vivo* is conflicting. When this drug was reviewed for its cytotoxicity and chromosomal damage in Wistar rats by Single cell gel (comet) assay, it was found to produce DNA damage in liver, kidney, urinary bladder and leukocytic cells [37]. Ribeiro *et al* in 2004 also reported DNA damage in rat peripheral leukocytes and oral mucosal cells, but they did not appreciate any chromosomal break down by CHX [38]. Buccal mucosal DNA damage due to Chlorhexidine use is also reported in human subjects, but the effect was found to be transient and reversible. [39] On the other hand Mohammadi *et al* in 2009 reported that in the clinically used concentrations, the biocompatibility of CHX are acceptable [40].

In the present study 0.2% CHX was used as it was the most commonly used in previous studies [28, 41]. Another most commonly available CHX formula is 0.12% however no difference has been reported in the clinical effect of 0.12% CHX and 0.2% CHX when it is used in optimal dosage. [24] In our study the whole study population did not show any significant difference in micronuclei count before and after use of CHX mouth wash (Table: 1). The Micronuclei evaluation was compared separately in male and females before and after (Table: 2) use of the drug and no significant sex differences in Micronuclei count was observed. Our finding was in accordance with the report of I Ros-Llor *et al* in 2014, who reported no genotoxicity using 0.12% CHX for 15 days in human trials [42]. On the contrary, Erdemir *et al* in 2007 reported increased Micronuclei count in human buccal mucosal cells by 0.2% CHX when used twice daily for 1 week [43]. But, they have used CHX for only 1 week and Micronucleus staining was done by using Giemsa stain. The increased micronuclei count in their study could be attributed to DNA nonspecific staining method. Holland *et al* in a paper in 2008 reported that to minimize the errors in MN count DNA specific stains should be used [21] and so in this study we used DNA specific stain Acridine orange (Loba Chemie, India).

Most studies determining the cytotoxicity of CHX have been done in the cell culture models and animals but human studies are few. *In vivo* and *in vitro* studies reporting genotoxicity of CHX have been done for a very short duration such as 4 wks [36-38, 42] Long term human studies evaluating micronucleus to assess the genotoxicity of CHX are not available in the literature. For our study, CHX had been used for a long period (9 weeks) following the report of Gu¨rgan *et al* in 2006 [11] criteria that may be helped the subjects to overcome the transient cytotoxic effect of the drug on oral mucosa by activating DNA repairing system [44]. There may be chance that in long term use on oral cavity CHX reacts differently in comparison to cultured cells in terms of cytotoxicity and this resistance can be attributed to some protective factors in the saliva [45]. In the present study, samples were collected only at the end of 9 weeks. However, sample collection at a regular weekly interval would probably be more appropriated to appreciate the possible changes in the buccal cells and to evaluate whether DNA repair mechanisms have a role in the process. In this study we also tried to note the objective and subjective symptoms of CHX mouth wash use. Some of the subjects had complained about burning sensation for initial days, but that had been reduced in later days. Reports are available related to mucosal thickening and dysplasia caused by CHX [17]. The findings of the present study was substantiated by the study conducted by Mackenzie *et al* in 1976 who did not observe any significant visible mucosal changes like mucosal thickening or keratinization after the use

of 0.2% CHX even after more than 1 year in human clinical trial.

4.1. Tables and Figures

Table 1: Mean Micronuclei count before and after using Chlorhexidine Gluconate mouth wash by the whole population

Treatment stage	Micronucleus count Mean (SD)	*P-Value
Before	0.7(0.65)	0.342
After	0.8(0.78)	

*P-Value for Wilcoxon-sign rank test was found to be not significant

Table 2: Mean Micronuclei count before and after using Chlorhexidine Gluconate mouthwash in male and female patients

Sex	Micronuclei count Mean (SD) before CHX Use	Micronuclei count Mean (SD) after CHX Use	*P-value
Male	0.5(0.52)	0.6(0.57)	0.586
Female	0.6(0.87)	0.7(0.78)	0.586

*****P-Value for Mann-Whitney U test was found to be not significant for both male and female.

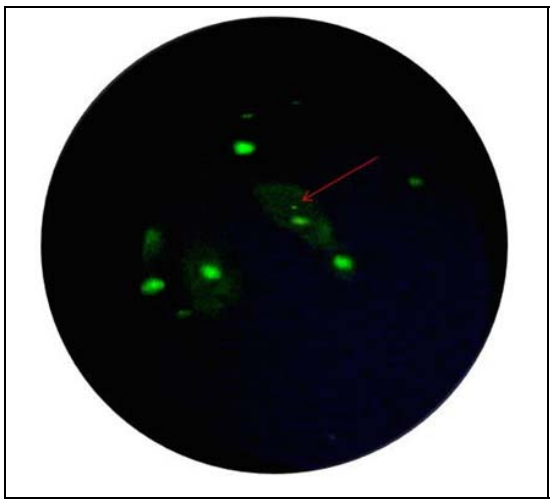

Fig 1: Photomicrograph showing buccal exfoliated cells (100X magnification) stained with Acridine Orange. The Micronuclei is marked with red arrow placed in close proximity to the main nucleus.

5. Conclusion

CHX mouth wash when used within pharmacological dose limit and duration of 9 weeks had no cytotoxic effect on the exfoliated buccal mucosal cells.

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