Dissertation

ALTERATIONS IN ORAL MUCOSAL CELLS USING ALCOHOL FREE AND ALCOHOL CONTAINING MOUTH-RINSES: A CLINICO-CYTOLOGICAL STUDY

Submitted to

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In the partial fulfilment of the requirements for the degree

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In

PERIODONTICS

By

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Under the guidance of

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BABU BANARASI DAS COLLEGE OF DENTAL SCIENCES

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(Faculty of Babu Banarasi Das University)

BATCH: 2015-2018

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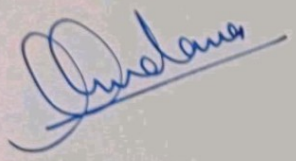

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DEDICATED TO

MY MOTHER

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"Better than a thousand days of diligent study is one day with a great teacher."

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ABBREVIATIONS

AM	Alcohol mouth rinse
AFM	Alcohol free mouth rinse
MN	Micronucleus
CA	Chromosomal aberration
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide)
NRU	Neutral red uptake
GI	Gingival index
PI	Plaque index
CHX	Chlorhexidne

Abstract

Over a period of more than three decades there has been quite intense interest in the use of chemical agents to control plaque and thereby gingivitis. Concern has been raised regarding the potential for alcohol-containing rinses to cause adverse effects when compared with alcohol free mouth rinse. So we decided to carry out a study with a larger sample size to know whether and to what extent commercially available mouth-rinses containing alcohol i.e Listerine and Eludril and alcohol free mouth rinses i.eHexidine and Rexidine Plus, have direct effect or not at clinical, genetic and cellular level of the oral mucosa.All 120 volunteers received primary phase of non surgical treatment including oral hygiene instructions and scaling, followed by use of mouth rinses for 2 month for the entire group as per the recommendations of the product. Statistical analysis demonstrated, the level of plaque index and gingival index for all four mouth rinses. Both indices showed significant reduction but the difference between the two groups was not significant. Clinically oral mucosal parameters i.e. epithelial desquamation, ulceration and petechiae were not seen after 2 months of use of the any of the two groups of mouth rinses. The cytological changes viz. cytotoxicity was assessed by using MTT Assay, NRU Assay and Trypan Blue Assay whereas DNA damage was assessed by using Micronucleus Assay and Chromosomal Aberration Assay. These assay demonstrated that the changes in the cells after 2 months use of Alcohol mouth rinse (AM) and Alcohol free mouth rinse (AFM) do cause cell damage but has not reached to the level of cytotoxicity or genotoxicity. This study, thus demonstrated that that use of Alcohol mouth rinse and Alcohol free mouth rinse in patients with gingivitis and mild periodontitis may result in improvement in clinical parameters, with absence of oral mucosal changes, and any cytological changes after 2 months.

Introduction

Dental plaque is defined clinically as a structured, resilient yellow –grayish substance that adheres tenaciously to the intra oral hard surfaces, including removable and fixed restorations. Dental plaque is the main etiologic agent in the development and progression of gingival and periodontal disease. Over a period of more than three decades there has been quite intense interest in the use of chemical agents to control plaque and thereby gingivitis. Chemical plaque control agents have been the subject of many detailed reviews since 1980. [1-3]

Numerous mouthwashes contains Actives, notably fluorides which are used for caries prevention[4], while Chlorhexidine(CHX) and cetylpyridinium chloride (CPC) ,are used for plaque control which inhibit the microbial growth.[5]Mouth rinses can be used as an adjunct to mechanical oral hygiene in conditions like: after subgingival scaling and root planing , in patients having inadequate oral hygiene, post scaling cervical hypersensitivity and also implicated to replace normal tooth brushing in various conditions like : after periodontal surgical procedures, intermaxillary fixation, during acute oral or gingival infection , for mentally or physically handicapped patients and in post-surgery patients.[6] Plaque control is the main method for preventing periodontal and dental diseases, however most available mouth-rinses are also significant sources of alcohol which is present as excipient in a varying amount (up to 27%) in mouth-rinse formulations.[7] Various studies have focused on alcohol – related side effects of mouthwashes containing alcohol. These clinical studies reported that the major side effects of alcohol – containing mouth-rinses include the presence of oral pain[8], burning sensation,[9] oral mucosal hypersensitivity and also epithelial epithelial desquamation[10], ulcerations, petechiae.[11] Contrary to it, few recent studies have been conducted which did not observe any cytological adverse effects when alcohol containing mouthwashes were compared to alcohol free

mouthwashes.[12-13] Some constituents of mouth rinse are known to be cytotoxic and genotoxic, thus causing chromosomal aberrations and DNA damage which severely irritate the mucosal surfaces of the mouth.[14] In the last few years, the interest for oral cytology as a diagnostic and prognostic methodology, for monitoring oral soft tissue aberrations and oral cancer has re-emerged substantially ,in which the presence and increase in number of Micronuclei (MN) in buccal mucosal cells represent genomic damage.Various studies have concluded that the gradual increase in MN counts in normal oral mucosa suggested a link between MN and malignant disorders.[15]

Concern has been raised regarding the potential for alcohol-containing rinses to cause adverse effects when compared with alcohol free mouth rinse.[16]

After through search of literature it was found that few studies show adverse effects of alcohol containing mouth washes on oral mucosa and few studies didn't show any such adverse effects. Such contrasting results could not give conclusive results regarding the effect of alcohol present in various mouthwashes on oral mucosa.

So we decided to carry out a study with a larger sample size to know whether and to what extent commercially available mouth-rinses containing alcohol and alcohol free have direct effect, if any,on the clinical, genetic and cellular level of the oral mucosa.

Aims & Objectives

AIM

To evaluate oral safety effect of commercially available mouth-rinses containing alcohol and alcohol free at their recommended doses.

OBJECTIVES

1. To analyze possible mucosal clinical changes at baseline and after 2 month of using Alcohol and Alcohol free mouth rinse .
2. To observe the effects of Alcohol and Alcohol free mouth rinse on plaque index and gingival index at baseline and after 2 month.
3. To assess possible cytological changes (cytotoxicity and DNA damage) after 2 months of alcohol and alcohol free mouth rinse.

Review of Literature

Rothenstein AS, Picozzi A, Doyle JL, Cancro LP , Singer EJ (1978) planned high frequency use of experimental cetylpyridinium chloride and commercial mouthwashes (scope, Listerine) to assess the possible irritant effects on the oral soft tissue under stringent test conditions. A double blind design used and soft tissue effects were measured over two week and recorded the occurrence of erythema, hyperemia, inflammation, keratosis, tongue coating and ulceration.[17]

Mark L. Bernstein, D.D.S., Louisville, Ky(1978) presented case report on oral mucosal white lesions associated with excessive use of Listerine mouthwash.Essential oils, astringents, and antiseptics are usually implicated in the etiology of hypersensitivity which is manifested by erythema, ulceration, or epithelial sloughing.The excessive topical application of Listerine mouthwash was found to be associated with asymptomatic, diffuse oral white lesions in two patients. The lesions, which were non ulcerated and did not scrape off, disappeared 2 weeks after Listerine lavage was discontinued.Features of an allergic response were present, and it was hypothesized that it was either due to cellular damage or an adaptive cellular response to minor irritation from one or more of the constituents of the mouthwash.[18]

J.M Gordon, I.B Lamster, M.C Seiger (1985) performed on Efficacy of Listerine antiseptic in inhibiting the development of plaque and gingivitis A 9-month double-blind controlled clinical study was conducted on adult subjects using either Listerine antiseptic, its vehicle control, or a water control in order to determine the efficacy of the antiseptic mouth rinse in inhibiting the development of plaque and gingivitis and Results demonstrated that Listerine antiseptic

significantly reduced the development of plaque at 1,3.6 and 9 months and the development of gingivitis at 9 months, as compared to its vehicle control or water control.[19]

DePaola LG, Overholser CD, Meiller TF, Minah GE, Niehaus C (1989) evaluated chemotherapeutic inhibition of supragingival dental plaque and gingivitis development, A 6-month double-blind, controlled clinical study was conducted on 107 healthy adult subjects to determine the efficacy of a mouthrinse used as a supplement to regular oral hygiene measures on supragingival dental plaque and gingivitis. Soft tissue, gingivitis, plaque area and extrinsic stain were evaluated again at 3 and 6 months. Results demonstrated that after 6 months, listerine produced a 34% inhibition of both plaque and of gingivitis.[20]

Deborah M. Winn, William J. Blot, Joseph K. McLaughlin, Donald F. Austin, Raymond S.(1991) evaluated on mouthwash use and oral conditions in the risk of oral and pharyngeal cancer. Interviews with 866 patients with cancer of the oral cavity and pharynx were included and 1249 controls of similar age and sex from the general population in four areas of the United States revealed increased risks associated with the regular use of mouthwash. Risks of oral cancer were elevated by 40% among male and 60% among female mouthwash users. Risks among both sexes generally increased in proportion to duration and frequency of mouthwash use. and summarized that this large population-based case-control study, showing little effect of oral hygiene factors, suggests that the regular use of mouthwash with high alcohol content contributes to oral cancer risk. According to them although the findings were consistent with the well-established risk associated with alcohol drinking, further they emphasized to clarify the results observed with mouthwash use.[21]

Kayo Koyama and Hirotsugu Yamamoto(1997) pilloted a study on effects of mouthwash on the human oral mucosa, there have been no fundamental studies on the influence of mouthwashes on the human oral mucosae. The effects of mouthwash was assessed with the use of exfoliative cytological and cytomorphometric analyses of smears obtained from clinically normal upper labium and cheek mucosa before mouthwashing, 30 sec, 10 min and 1 hr after mouthwashing. The oral mucosae of smokers were more irritated by use of mouthwash than that of the never-smokers. After 1 hr of mouthwash, there was decrease in the nuclear and cytoplasmic areas of cells and increases in inflammatory cells. So this study concluded that more attention should be paid when mouthwashes were used as daily oral hygiene regime.[22]

Jones CG (1997) Chlorhexidine: is it still the gold standard?After 20 years of use by the dental profession, CHX is recognized as the gold standard against which other antiplaque and gingivitis agents are measured. CHX is antiplaque effect is a result of the dicationic nature of the CHX molecule, which affords the agent the property of persistence of antimicrobial effect at the tooth surface, through both bactericidal and bacteriostatic effect. By understanding how the chemical properties of the CHX molecule can explain the plethora of clinical efficacy and safety data, the use of CHX can be optimally aimed towards the patient groups who would most benefit from the superior therapeutic effect of the agent. Specifically, CHX would seem to be of most value to patients in whom the ability to perform adequate oral hygiene procedures has been compromised.[23]

Gurgan S, Onen A, Kuprıslı H(1997)evaluated in vitro effects of alcohol-containing and alcohol-free mouthrinses on microhardness of some restorative materials. Eighteen cylinders of each restorative were fabricated and initially

stored in distilled water for 24 h. Six samples of the restoratives were stored for 12 hours to simulate a 2 min/day for 1 year exposure to mouthrinses in the following solutions: distilled water (control), alcohol-containing mouthrinse (Viadent) and alcohol-free mouthrinse (Rembrandt). At the end of the test period microhardness was measured with a Tukonmicrohardness tester. Kruskal-Wallis one-way analysis of variance was used to analyse the data. Both alcohol-containing and alcohol-free mouthrinses similarly affect the hardness of the materials tested.[24]

Moghadam BK, Gier R, Thurlow T (1999) discussed on extensive oral mucosal ulcerations caused by misuse of a commercial mouthwash This study describes severe mucosal injuries following misuse of an undiluted over-the-counter mouthwash with a high alcohol content (70%), oil of peppermint and arnica.The mouthwash was to be diluted 5:1 with water. The patient used undiluted solution to better treat her self-diagnosed "contagious gum infection." She experienced burning sensation with each rinse and developed severe mucosal injuries subsequently. Her oral condition improved within 48 hours following discontinuation of use of the mouthwash and application of a mixture of Benadryl Elixir, Maalox Plain, and 2% viscous Lidocaine.[25]

Norppa H, Falck GC(2003) reviewed a description on what do human micronuclei contain?As micronuclei (MN) derive from chromosomal fragments and whole chromosomes lagging behind in anaphase, the MN assay can be used to show both clastogenic and aneugenic effects. The distinction between these phenomena is important, since the exposure studied often induces only one type of MN. This particularly concerns the use of MN as a biomarker of genotoxic exposure and effects, where differences in MN frequencies between exposed subjects and referents are expected to be small.Understanding the mechanistic

origin and contents of MN is essential for the proper use of this cytogenetic end-point in biomarker studies, genotoxicity testing and risk assessment.[26]

Ciancio S (2003) piloted a study on Improving oral health: current considerations The high incidence of periodontal disease among adults in the Western world indicates that in most cases, routine dental care could be considerably improved. The progressive effect of the disease suggests that improvements in oral cleanliness are mandatory if large numbers of adults are to retain their teeth into old age. Data show that periodontal disease can be minimized through effective plaque control, and that a combination of brushing, interdental cleaning, and chemotherapeutic agents (e.g. mouthwash) is beneficial to patients with plaque control problems.[27]

Poggi P, Baena RR, Rizzo S and Rota MT in(2003) reviewed the cytotoxic effects of mouth rinses with Alcohol on Human Gingival Fibroblasts in Vitro and the effect of its intermediate Acetaldehyde on Human Gingival Fibroblasts (HGFs). Cultured HGFs were exposed to different concentration of acetaldehyde and cell viability was evaluated on third and fifth day of incubation. They resulted that acetaldehyde produced a dose and time dependent inhibition on Cell Adhesion and Viability , together with Disruption of cytoskeleton structures and cytoplasm organelles.[28]

Kristen U, Friedrich RE(2004) performed a study on Toxicity screening of mouthwashes in the pollen tube growth test: safety assessment of recommended dilutions of twenty brands.In this study the irritation of the oral mucosa was examined after dilution of mouthwash as recommended by the

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brands were examined by the pollen tube growth test, an alternative in vitro method for the irritation assessment of ingredients of cosmetic formulations. The test was based on the photometric quantification of pollen tube growth inhibition. The parameter was expressed by IC50 values which characterized the cytotoxic potency of a product. The IC50s clearly revealed that none of the tested mouthwashes should cause acute irritation if used at the recommended dilution. However, 4 to 5 of the mouthwashes could probably irritate the oral mucosa acutely, when used in the form of the original producer concentrations .The limitation of this study was that they did not focus on the various components of the mouthwashes which actually lead or not lead to oral irritation.[29]

Camila Lopes Cardoso, RenataFalchete do Prado, LuisAntonio de AssisTaveir (2005) compiled a study on Macroscopic and microscopic study of tissue response to oral antiseptics and its influence on carcinogenesis. This study aimed at conducting a macroscopic and microscopic analysis of the tissue response of tongue mucosa of hamsters to daily topical applications of antiseptics (Anapyon, Listerine, Oral B) during 13 and 20 week.Three serial sections of each tongue were evaluated, and characteristics related to epithelial hyper keratinization, atrophy, hyperplasia and dysplasia were organized . Despite the observation for moderate dysplasia in one case in the Anapyon 20 week group, the further results were very similar to the control group (saline solution), eliminating the need of comparative statistical tests. By means of such methodology for testing the carcinogenesis-initiating action, it was concluded that oral antiseptics are unable to trigger the development of neoplasms.[30]

Barnett ML(2006) performed a review on the rationale for the daily use of an antimicrobial mouthrinse along with mechanical plaque control methods.The author reviewed studies demonstrating the essential etiologic role of a pathogenic dental plaque biofilm in the development of gingivitis, as well as studies indicating that most people fail to maintain a level of mechanical plaque control sufficient to prevent disease. In addition, he did a brief review of studies of oral microbial ecology that identified the oral mucosal tissues as a reservoir of bacteria that colonize tooth surfaces, and he summarized six-month clinical studies of marketed antimicrobial mouthrinse ingredients and products. And concluded that daily use of an effective antiplaque/antigingivitis antimicrobial mouthrinse is well-supported by a scientific rationale and can be a valuable component of oral hygiene regimens.[31]

Bernstein ML, CarlishR(2007)accompalished a study on Mouthrinses containing alcohol and oral cancer reports.The strong association between alcohol usage and the development of oral cancer (OC) was reported in numerous papers. As some mouthrinses contain significant amounts of ethanol, a possible relationship to pathology had been considered. The purpose of the present paper was to analyze several epidemiological studies which evaluated the association between commercial mouthrinses and the etiology of OC but controversial aspects made it difficult to find a clear relationship between alcohol-containing mouthrinses and OC.[32]

Mala Kamboj ,Sumita Mahajan(2007) concluded that Micronucleus—is an upcoming marker of genotoxic damage ,Micronucleus assay was performed on oral exfoliated cells of chosen subjects having leukoplakia and squamous cell

ChlorhexidineGluconate) and Tanflex (0.15% Benzydamine HCL) on buccal epithelial cells by MN test. 28 patients with aged 16-24 underwent three mouth rinses and analyzed before and after one week exposure.Physiologic saline was used for the control group. The micronucleus incidence was scored in the Buccal epithelial of each participant.The results of this study showed that although the micronuclei incidence increased in Klorhex, Tanflex and Andorex groups after exposure to mouthrinses, but the micronuclei incidence of Klorhex and Tanflex groups increased, except Andorex, when compared with the control group. Now although there was differences between both Klorhex and the control groups and Tanflex and the control groups,but there was not any difference between Andorex and the control groups. Hence they showed that cytotoxicity mechanism could be produced in a time- and CHX concentration- dependent manner. This reason can be contributed to this result, as lowered concentration of CHX exists in Andorex, the combinations of CHX and Benzydamine HCL can lessen the cytotoxicity of this mouthrinse.[35]

McCullough MJ, Farah CS (2008)focussed on role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes It has been long established that smoking and alcohol consumption are risk factors linked to the development of oral cancer. This review assesses the epidemiological evidence, supportive in vitro studies and mechanism by which alcohol is involved in the development of oral cancer. Further, they reviewed the literature that associates alcohol-containing mouthwashes and oral cancer. On the basis of this review, they believed that there was now sufficient evidence to accept the proposition that alcohol-containing mouthwashes contribute to the increased risk of development of oral cancer and further felt that it is inadvisable for oral

healthcare professionals to recommend the long-term use of alcohol-containing mouthwashes.[36]

Silverman S Jr, Wilder R.(2008) conducted a study on Antimicrobial mouthrinse as part of a comprehensive oral care regimen. Safety and compliance factors,The authors reviewed studies relating to the safety and efficacy of alcohol-containing mouthrinses, as well as studies indicating that most patients fail to comply with oral health care recommendations and concluded that Alcohol-containing antimicrobial mouthrinses are safe and effective as part of a daily oral care regimen to prevent or minimize periodontal disease. However, many patients do not comply with instructions on how to use them.[37]

Celso Augusto Lemos-Jьnior; GermanoEduardo,MiguelVilloria (2008) Reviewed evidence about the safety of the daily use of alcohol-based mouthrinses and this Current scientific knowledge provides clear evidence that alcohol-based mouthwashes can be beneficial in a daily oral health routine, including dental hygiene and plaque control. Several issues are worth discussing, in spite of the wealth of supporting evidence. According to this review despite some undesirable effects to some people, like burning sensation, and some contraindications, like the use by infants, alcohol addicts and patients with mucosal injuries, there is no reason to avoid the use of alcohol-containing mouthwashes as long as they are used following proper guidance by dental professionals and the manufacturers' instructions.[38]

Muhammad WasifHaq, MehwishBatool, Syed HammadAhsan, Navid Rashid Qureshi(2009) evaluated Alcohol use in mouthwash and possible oral health concerns, its Objective was to establish the presence and quantify Ethanol

in commercially available mouthwashes, The concentration of alcohol used in the mouthwash lags behind the optimum concentration of 50% to 70% at which alcohol is able to exert its antiseptic effect, hence except for its use as a solvent, alcohol in the mouthwash does not contribute to any other therapeutic effect. Due to this reason, alcohol free mouthwashes in the clinical trials have proven to be as effective as alcohol based mouthwashes, with the former having lesser side effects [39]

Werner CW, Seymour RA(2009) conducted a study are alcohol containing mouthwashes safe? Dentists need to be aware that there is a hypothetical risk for the development of oral cancer from repeated use of alcohol containing mouthwashes.This study critically evaluated and explored the data on the efficacy of the addition of alcohol to mouthwashes. Alcohol (ethanol) is a constituent of many proprietary mouthwashes. The evidence suggests that the alcohol component of mouthwashes affords little additional benefit to the other active ingredients in terms of plaque and gingivitis .[40]

Gunsolley JC (2010) recorded clinical efficacy of anti-plaque, anti-gingivitis mouth rinse. And graded that mouth rinse with Chlorhexidine as an active agent are effective anti-plaque, anti-gingivitis agent .The evidence evaluated from systematic reviews of six months clinical trials and resulted mouth rinses with cetylpyridinium chloride (CPC) as an active agent was weaker due to few clinical trials testing the same formulation of CPC. Delmopinol was an effective anti-plaque, anti-gingivitis agent.[41]

Isabel Lanzys David Herrera ,Sagrario Santos (2011) assessed on the microbiological effects of an antiseptic, non-alcohol based mouth-rinse containing chlorhexidine and cetylpyridinium chloride, in patients undergoing radiation

therapy for head-and-neck cancer. . Cancer patients were randomly assigned to one of the two treatments (test mouth-rinse or a placebo). Three visits were scheduled (baseline, 14 and 28 days). Microbiological findings were evaluated in tongue, mucosa and subgingivalsamples, by means of culture.The detection of Candida species in mucosa and tongue samples showed significant reductions in the test group. Total bacterial counts decreased in both groups from baseline to the 2-week visit, while minor changes occurred between 2 and 4 weeks (effects onP. gingivalis, P. intermedia, C. rectus, E. corrodens). , this study suggested that the use of the tested mouth-rinse may lead to improvements in microbiological parameters in patients irradiated for head-and-neck cancer.[42]

Koschier F, Kostrubsky V, Toole C, Gallo MA (2011)studied ,In vitro effects of ethanol and mouthrinse on permeability in an oral buccal mucosal tissue construct.The current study investigated the influence of ethanol and ethanol-containing mouthrinses on model chemical permeability in an in- vitro oral buccal mucosal construct.. Caffeine flux in buccal tissue was measured after pre-treatment with ethanol or Listerine products under conditions modeling a typical mouthwash rinsing.Specifically, a 30sec exposure to alcohol products followed by a 10hr non-treatment phase and then a 30sec exposure prior to addition of caffeine. At 10min specific intervals, media was collected from the buccal tissue ,for analysis of caffeine.The results demonstrated no increase in caffeine flux due to prior exposure to either ethanol or Listerine, No cytotoxicity or histopathological effects were observed in these tissues.[43]

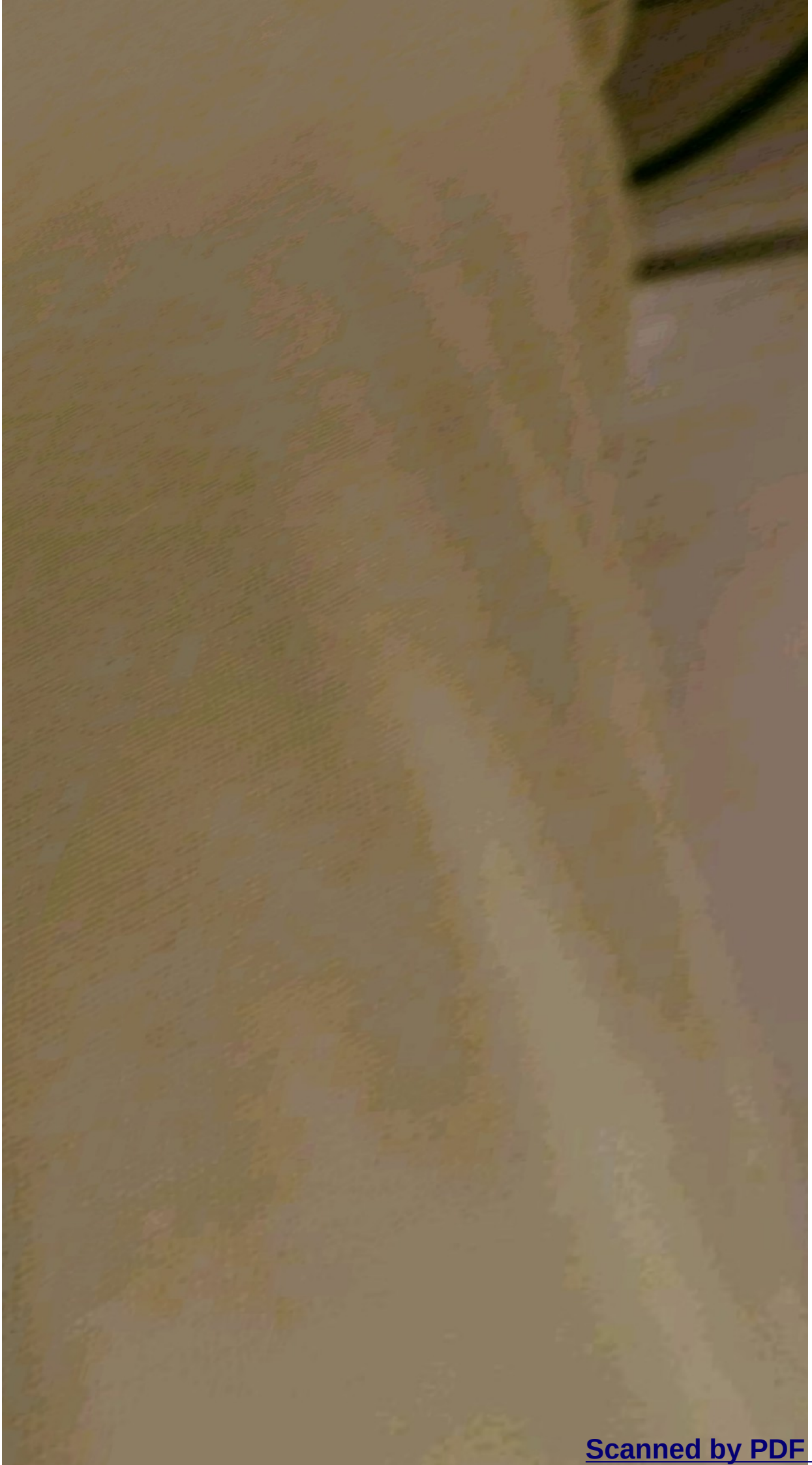
Reidy JT, McHugh EE, Stassen LF(2011) reviewed on the role of alcohol in the pathogenesis of oral cancer and the link between alcohol-containing mouthrinses and oral cancer .The article reviewed the most recent literature on

the effects of alcohol on the oral mucosa, and the possible mechanisms by which alcohol is thought to act as a carcinogen.. Evidence regarding the carcinogenic effect of alcohol-containing mouthrinses is inconsistent, and a link between the use of alcohol-containing mouthrinses and the development of oral cancer has not yet been firmly established.[44]

Turkez H, Togar B, Arabaci T(2012) The present study was undertaken to investigate the in vitro genotoxic potential of Listerine(LN) using micronucleus and single cell gel electrophoresis tests as genetic endpoints.The result of the present study showed that there were no statistically significant differences between the control group and the groups treated with LN alone in both analysed endpoints. In conclusion, the results demonstrated the absence of genotoxicity of LN on human lymphocytes.[45]

Carlin V, Matsumato MA, Saraiva PP, Artoli A, Oshima CT, and Ribeiro DA in (2012) compiled a study to see cytogenetic damage induced by mouth rinses formulations in vivo and in vitro. A total of 75 volunteers were included in the search. Exfoliated Buccal mucosa was collected and micronucleus test was used to evaluate mutagenenicity and cytotoxicity in vivo. They concluded that Chlorhexidine o.12% was able to induce cytogenetic damage in vivo and in vitro respectively whereas Listerine is an antioxidant agent. [46]

Goutham BS ,Manchanda K, Sarkar AD, Prakash R, Jha K, Mohammed S (2013)correlated the efficiency of two commercially available mouth rinses – Chlorhexidine and Listerine on Plaque and Gingivitis. A double blind study was done on 150 patients for 2 months, and reported reduced plaque growth and



gingival inflammation. However Chlorhexidine was more effective against plaque regrowth then the phenolic rinse. [47]

AL Zamora-Perez(2013) focused on Increased Number of Micronuclei and Nuclear Anomalies in Buccal Mucosa Cells From People Exposed to Alcohol-Containing Mouthwash.The aim of this study was to evaluate the effects of alcohol-containing mouthwash on the induction of micronuclei and nuclear anomalies in exfoliated buccal cells, including binucleated cells, cells with nuclear buds, and karyolitic, karyorrhectic, condensed chromatin, and pyknotic cells.Buccal mucosa cells were collected from 107 healthy participants who were divided into three groups: control subjects who did not use mouthwash (n = 33), subjects who were exposed for 30 days and two times rinsing with 30 seconds each time to alcohol-containing mouthwash (n = 38; 26% ethanol concentration); and subjects exposed to a non-alcohol-containing mouthwash (n = 36).And results suggest that subjects exposed to alcohol-containing mouthwash exhibited an increase in frequency of micronuclei and nuclear anomalies in oral mucosal cells, which is directly related to DNA damage.[48]

Madan PD, Sequeira PS, Shenoy K, Shetty J (2008) reported the effect of three mouthwashes on radiation-induced oral mucositis in patients with head and neck malignancies: a randomized control trial The present study was done to assess the effect of three alcohol-free mouthwashes on radiation-induced oral mucositis in patients with head and neck malignancies, Eighty patients with head and neck malignancies, scheduled to undergo curative radiotherapy, were randomly assigned to receive one of the three alcohol-free test mouthwashes (0.12% chlorhexidine, 1% povidone-iodine, or salt/soda) or a control, This study

demonstrates that use of alcohol-free povidone-Iodine mouthwash can reduce the severity and delay the onset of oral mucositis due to antineoplastic radiotherapy.[49]

IreneRos-Llor (2014) reviewed on Cytogenetic analysis of oral mucosa cells, induced by chlorhexidine, essential oils in ethanolic solution and triclosan mouthwashes.The aim of this study was to evaluate DNA damage and cytokinetic defects, proliferative potential and cell death caused by the frequent use of mouthrinses containing chlorhexidine, triclosan and essential oils in ethanolic solution, compared to a placebo mouthwash.This double-blind, prospective, randomized clinical trial included 80 Caucasian patients. Subjects were divided into four groups: Group I used a mouthrinse, Triclosan; Group II used physiological saline; Group III used chlorhexidine; Group IV a mouthrinse with essential oils in ethanolicsolution.The result did not observe any genotoxic effect resulting from mouthrinse use.[50]

R. Shashikala, A. P. Indira, G. S. Manjunath, K. Arathirao, and B. K. Akshatha (2015) performed this study to see the role of micronucleus in oral exfoliative cytology ,the interest for oral cytology as a diagnostic and prognostic methodology, for monitoring patients in oral potentially malignant disorders and oral cancer has re-emerged substantially. In 1983, buccal mucosal micronuclei assay was first proposed to evaluate genetic instability.Various studies have concluded that the gradual increase in micronucleus (MN) counts from normal oral mucosa to potentially malignant disorders to oral carcinoma suggested a link of this biomarker with neoplastic progression.Therefore, MN assay in exfoliated cells holds promise as a specific biomarker for exposure to various carcinogens, and can also be used as a screening test in oral health centers.[51]

Materials & Methods

Materials and Method

A 2 month longitudinal prospective clinical study was carried out in the Department of Periodontics, Babu Banarasi Das College of Dental Sciences (BBDCODS), Lucknow, to analyse possible mucosal changes at clinical and cytological level by the use of commercially available mouth rinses containing alcohol and alcohol free mouth rinse. A total of 120 volunteers were included in the research and were randomly distributed in to four groups Group A, Group B, Group C and Group D. Group A, Group B volunteers used mouth rinse with alcohol and Group C, Group D volunteers used mouth rinse without alcohol. The study was carried out in collaboration with Indian Institute of Toxicology Research (IITR), Lucknow for the analysis of cytological smears. An appropriate clearance from the Institutional Ethics Committee was taken for the study. Patients were clearly explained the study protocol and procedure. A duly signed written consent was taken from them.A strict inclusion and exclusion criteria was followed for the recruitment of the volunteers.

PATIENT SELECTION:

Medical and dental history was obtained at the time of screening . The selection criteria were based upon the following inclusion and exclusion criteria:

INCLUSION CRITERIA :

1. All Subjects in the age group of 25 – 45 years, irrespective of gender.
2. All Subjects coming to the OPD diagnosed with gingivitis as well as mild periodontitis.

EXCLUSION CRITERIA :

1. Pregnant, lactating and post-menopausal women.
2. Smokers, tobacco and/or pan masala chewers.

3. Any significant systemic disease.
4. Patients who are taking xerostomia drugs.
5. Patients who have used antibiotics for the past 3 months.
6. Non co-operative patients.
7. Systemic disease or condition which can result in to alteration of oral mucosa.

STUDY DESIGN:

The study was conducted in the Department of Periodontics, Babu Banarasi Das College of Dental Sciences ,Lucknow. It was a randomized,longitudinal study, where clinical and cytological changes were seen at baseline and at 2 months. Cytological processing for the analysis of cell viability was done at IITR. The enrolment of volunteers , their clinical examination and buccal scraping was done at BBDCODS, Lucknow.

GROUP DESCRIPTION:

After the diagnosis, volunteers were randomly divided in to 4 groups and each group includes 30 volunteers and distributed in to group Group A, Group B, Group C and Group D. Where Group A, Group B used alcohol containing mouth rinse and Group C, Group D used alcohol free mouth rinse.

Group A (n=30) : To rinse with Listerine [Johnson & Johnson]

Group B (n=30): To rinse with Eludril. [Win Medicare]

Group C (n=30) To rinse with Hexidine. [ICPA Health]

Group D (n=30) To rinse with Rexidine plus. [Warren Pharma.]

Gr A + Gr B = Alcohol containing mouth rinse **(AM)**

Gr C + Gr D = Alcohol free mouth rinse **(AFM)**

COMPOSITION OF LISTERINE

Active Ingredients	Inactive Ingredients
Thymol	Alcohol (21.6%)
Eucalyptol	Sorbitol
Methyl Salicylate	Sodium Saccharine
Menthol	Sodium Benzoate

COMPOSITION OF ELUDRIL

Active Ingredients	Inactive Ingredients
CHX digluconate	Alcohol (42.7%)
Chlorobutanol hemihydrate	Sorbitol

COMPOSITION OF REXIDINE PLUS

CHX gluconate (0.2%)
Sodium monofluorophosphate
Triclosan

COMPOSITION OF HEXIDINE

CHX gluconate (0.12%)
Saccharin
Glycerin
Peppermint
Purified water

CLINICAL PARAMETERS

Following clinical parameters were recorded at baseline and after 2 month:

- Gingival Index GI (Loe and Silness, 1963)
- Plaque Index PI (Silness and Loe, 1964)

ORAL MUCOSAL PARAMETERS

Following oral mucosal parameters were recorded at baseline and after 2 month:-

- **Epithelial desquamation:** It is a naturally occurring process in which the outer layer of mucosal cells is sloughed off.
- **Ulcerations :** It is a circumscribed inflammatory and often suppurating lesion on the mucosal surface resulting in necrosis of tissue.
- **Petechiae:** It is a small (1–2 mm) red or purple spot on the mucosal surface , caused by a minor bleed from broken capillaryblood vessels.

CYTOLOGICAL CHANGES

Cytological changes was assessed after 2 months use of AM and AFM by the help of

- MTT assay
- Neutral red uptake (NRU)
- Trypan blue dye exclusion assay
- Micronucleus (MN) assay
- Chromosomal Aberration (CA) assay

MATERIAL

Armamentarium for Diagnosis & Scaling & Root planing

Armamentarium for Buccal swab collection

- Wooden stick

Armamentarium For Sample Transportation

- DMEM/F-12 medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) tube
- Ice box

Armamentarium For Processing Of Buccal Swab:

- Tissue Culture Plates
- CO_2 Incubator
- Centrifuge Machine
- Cell Counter
- Microscope

PROCEDURE:

The treatment procedure was fully explained to the patient , detailed case history was recorded and a duly signed written consent was obtained from each patient before initiating the procedure.

PHASE I THERAPY

All the selected subjects underwent phase I therapy which included oral hygiene instructions, full mouth supra and sub gingival scaling and root planing. Scaling and

ARMAMENTARIUM FOR SCALING &ROOT PLANING

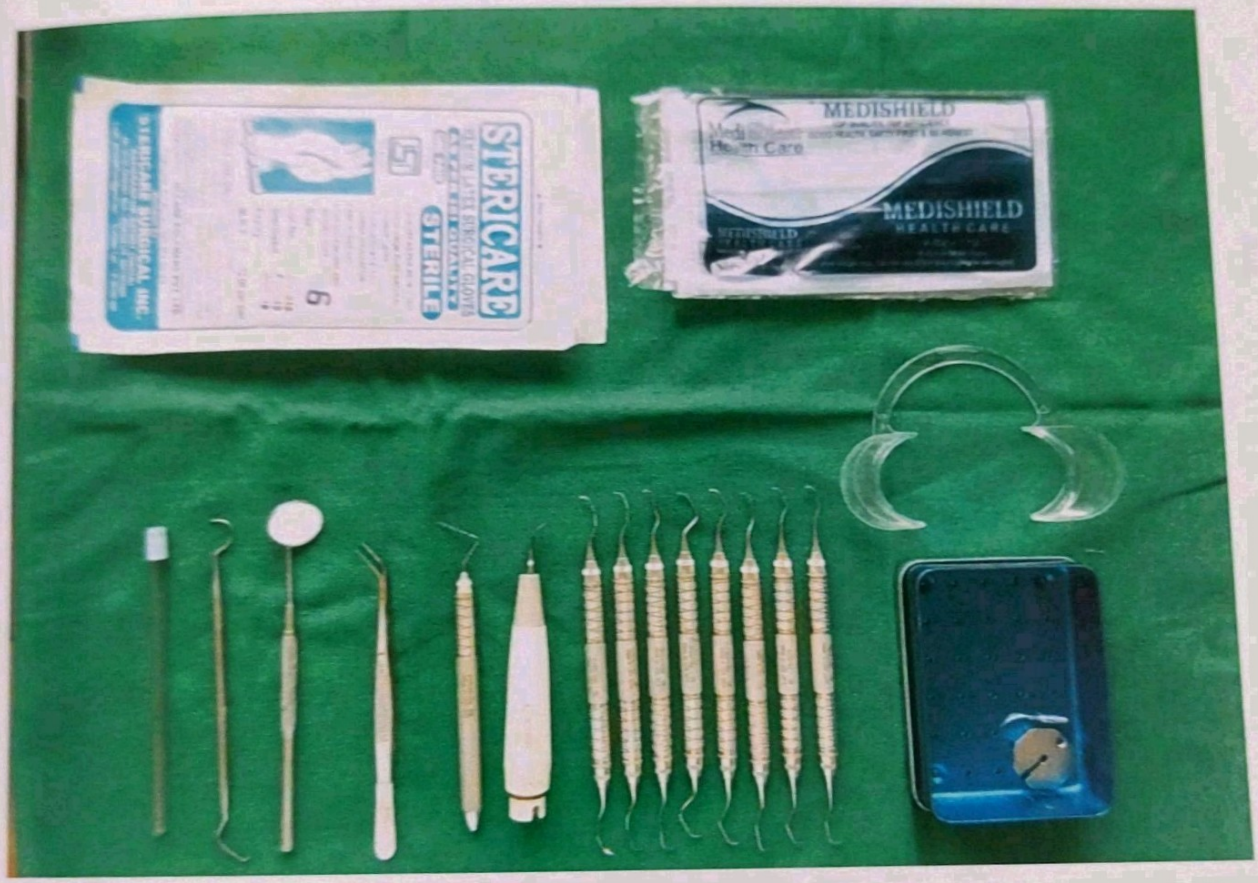


Plate No:- I

root planing were performed using hand and ultrasonic instruments. And randomly volunteers were prescribed to use mouth rinse for 2 months.

Buccal Swab Collection

Buccal swab collection of 120 volunteers of age group 25 – 45 years , were done at babu banarasi das college of dental science , lucknow.Before collecting the swab, we had confirm that the volunteers mouth is empty because if there is food, gum, or tobacco, the swab will be introduced to substances that could interfere with cytological procedures. We had placed a wooden stick on the side of the cheek and gently rubbed the cheek for about 5-10 seconds, the volunteers did not experience any pain. Immediately after swabbing, we placed the swab directly in to DMEM/F-12 medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) tubes, kept the samples in to ice box and transported it to IITR for its processing. Where the samples processed on same day by following procedures:

MTT assay:

Cytotoxicity assessment was done using standard endpoint i.e., tetrazolium bromide MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. In brief, cells ($1x10^4$ cells/well) were seeded in 96-well tissue culture plates and incubated in the CO_2 incubator for 24, 48, 72 and 96 h at 37° C. Tetrazolium salt (10 µl/well; 5 mg/ml of stock in PBS) was added 4 h prior to completion of respective incubation periods. At the completion of incubation period, the reaction mixture was carefully taken out and 200 µl of culture grade DMSO was added to each well. The content was mixed well by pipetting up and down several times until dissolved completely. Plates were then incubated for 10 minutes at room temperature and color was read at 550 nm using Multiwell Microplate Reader (Synergy HT, Bio-Tek, USA).

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ARMAMENTARIUM FOR BUCCAL SWAB COLLECTION

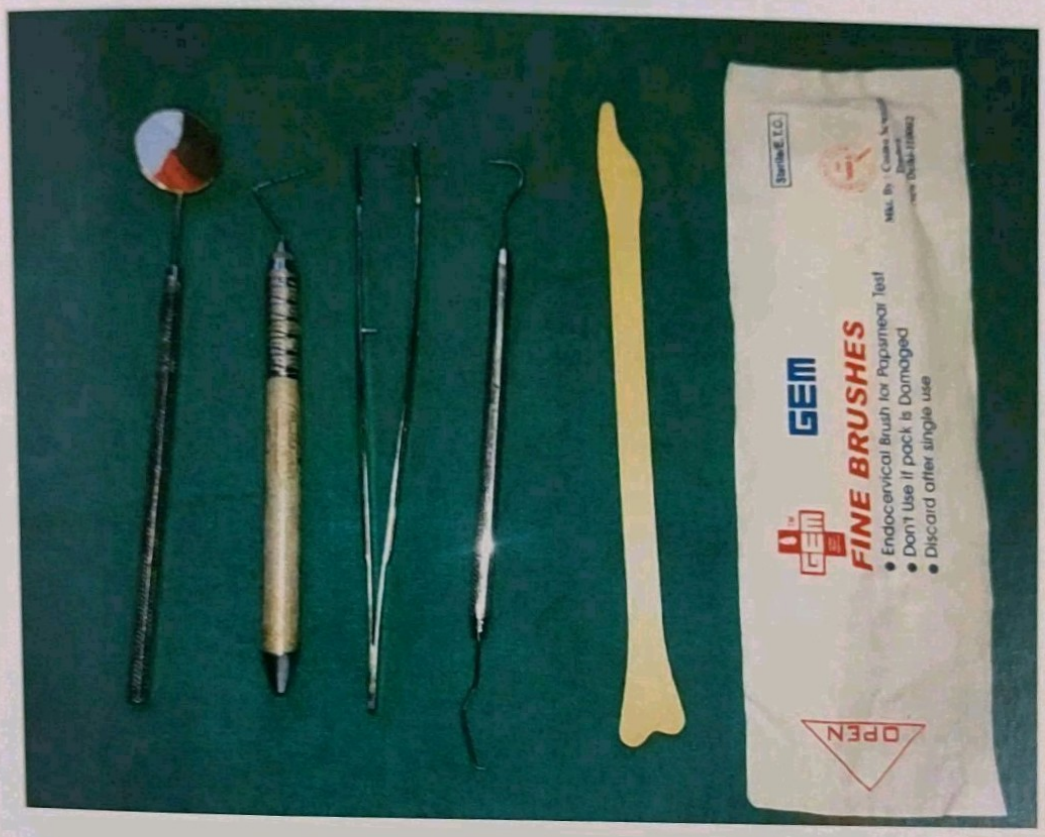
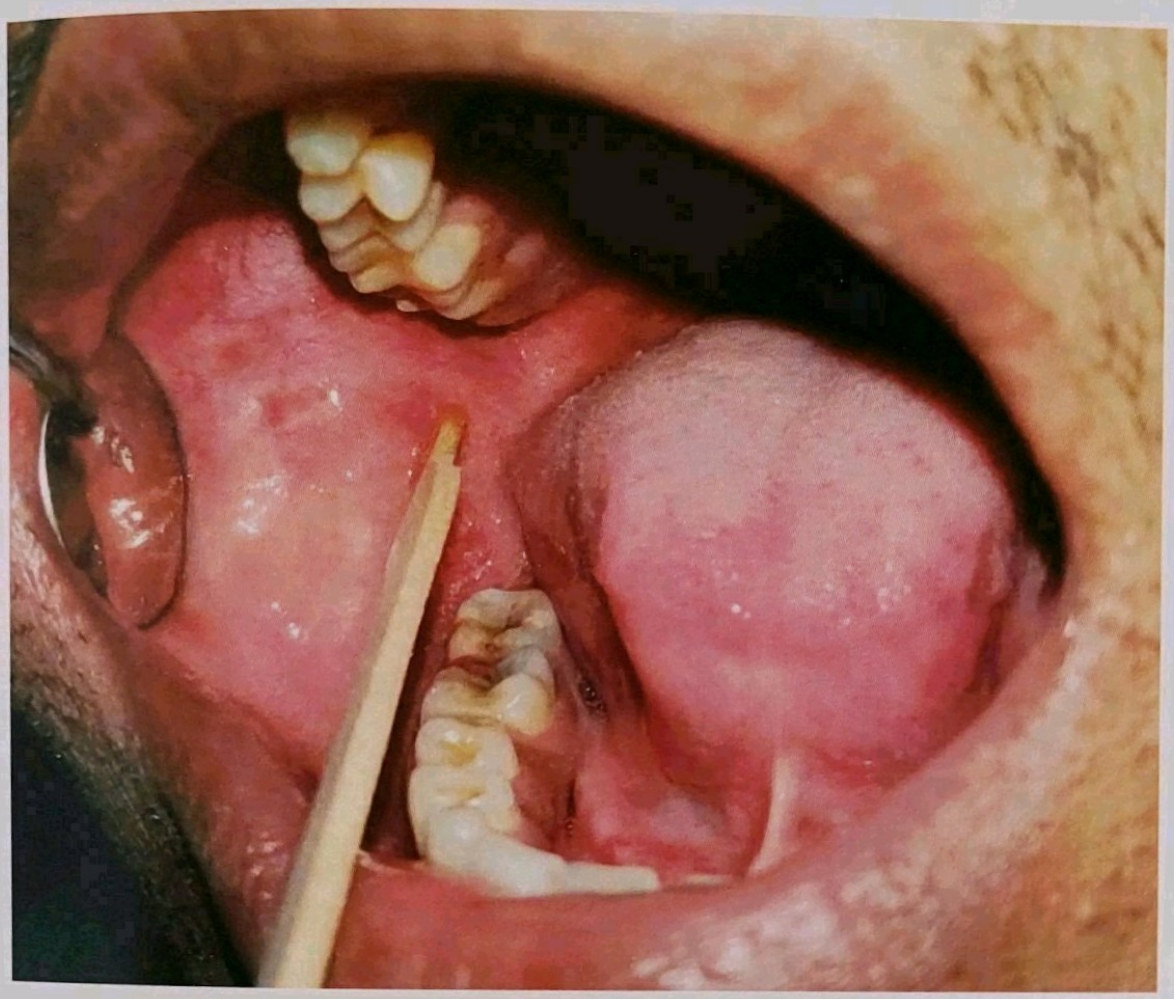


Plate No:- II

BUCCAL SWAB COLLECTION BY WOODEN STICK



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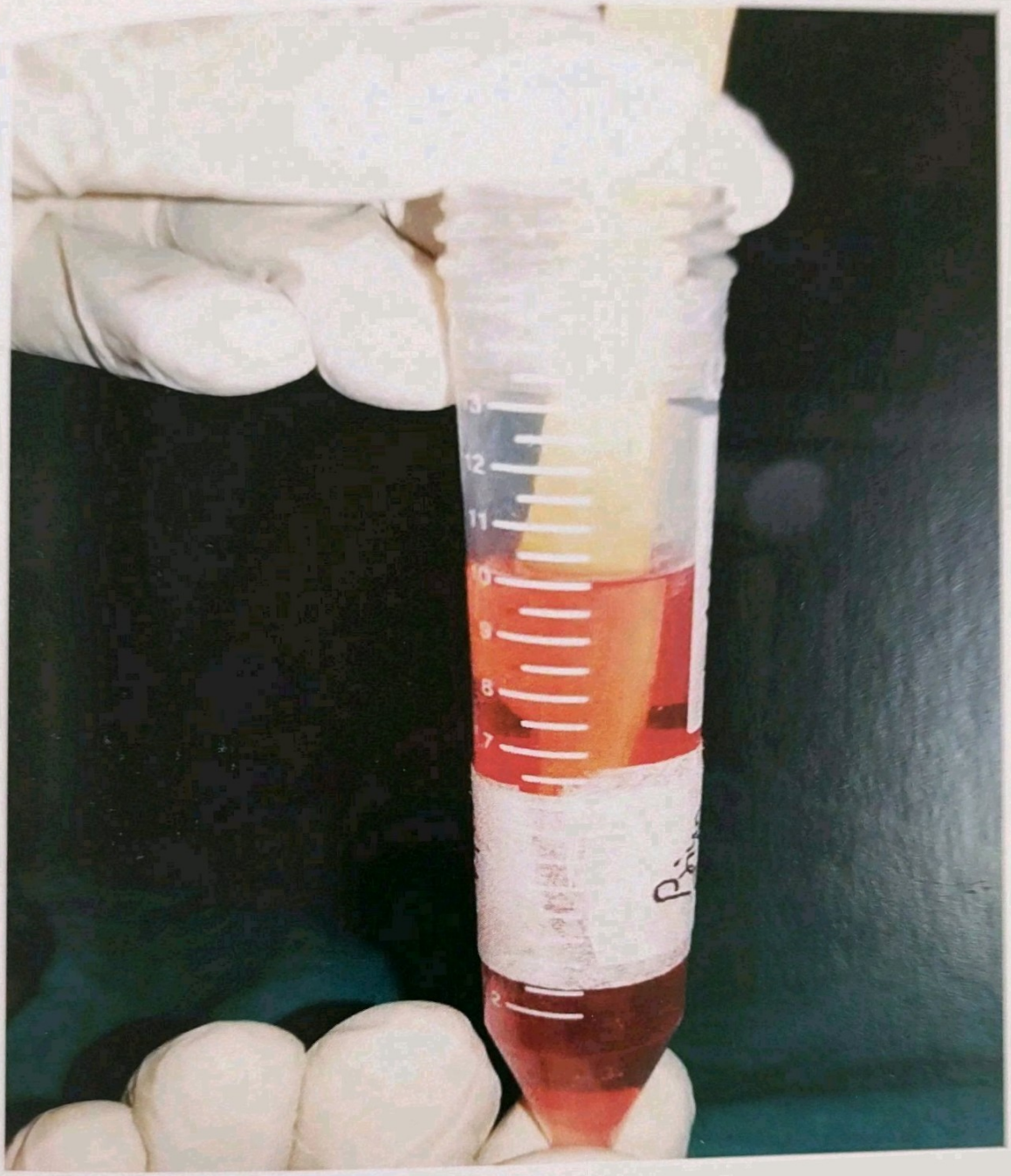
TRANSFERRING BUCCAL SWAB IN DMEM/F-12 MEDIUM



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The unexposed sets, and sets exposed to $MnCl_2$ (10^{-3} M) were also run parallel under identical conditions that served as a basal and positive control respectively.

Neutral Red Uptake (NRU) assay:

To ascertain the toxicity of the compound on cells Neural Red Uptake (NRU) assay was carried out was carried out. In brief, cells (1×10^5/well were seeded in poly-L-lysine (PLL) pre-coated 96-well culture plates and allowed to adhere for 24 h. Thereafter, the cells were exposed to variable concentration of MCP (30µM-400µM) and further incubated for a period of 24 h. At the end of incubation period the medium from the wells was aspirated out. After one was with 1XPBS NRU was added at a final concentration of 50ug/ml in medium. After 3 h incubation at 37°C, NRU was removed and cells were washed with 1% acetic acid and 50% ethanol. For fixation cells were incubated in 0.5% formaldehyde for 20 min at 37°C. Post incubation absorbance was taken at 540 nm, using a multi-well microplate reader (Synergy HT; Bio-Tek, USA). The experiment was run in triplicates. Untreated sets were run simultaneously under identical conditions and served as basal control.

Trypan Blue Dye Exclusion Assay:

The test was conducted to study the cell viability by assessing the loss of membrane integrity following the method of Pant et al., (2001). In brief, the cells (4×10^5/well) were seeded in 48 well culture plates and incubated for 24, 48, 72 and 96 h in 5% CO_2 - 95% atmosphere at 37°C under high humid conditions. The culture medium was replaced every alternate day. The cells were then subjected to assess the loss of cell viability. Immediately after the completion of respective time periods, cells suspensions were aspirated and centrifuged at 600 rpm for 5 min and washed twice with sterile PBS (pH 7.4), and re-suspended in a small amount of PBS. The cell

Observations & Results

Statistical analysis

Data were summarised as Mean ± SE (standard error of the mean). Groups were compared by repeated measures of two factor analysis of variance (ANOVA) and the significance of mean difference within (intra) and between (inter) the groups was done by Newman-Keuls test after ascertaining normality by Shapiro-Wilk's test and homogeneity of variance between groups by Levene's test. A two-tailed (α=2) $p<0.05$ was considered statistically significant. Analyses were performed on SPSS software (Windows version 17.0).

Results and Observations

The present clinico-cytological study evaluates alterations in oral mucosal cells using alcohol free and alcohol containing mouth rinses. Selected samples were randomised equally into four groups and treated with Listerine, Eludril which are alcohol containing mouth rinse; Rexidine plus and Hexidine which are alcohol free mouth rinse. The outcome measures of the study were oral mucosal changes, cytological changes and changes in clinical parameters . In oral mucosal changes epithelial desquamation, ulceration and petechiae were examined at baseline and after 2 months. The cytological changes viz. cytotoxicity was assessed using MTT Assay, NRU Assay and Trypan Blue Assay whereas DNA damage was assessed using Micronucleus Assay and Chromosomal Aberration Assay. The clinical parameter PI was assessed according to Silness and Loe, 1964 whereas GI was assessed according to Loe and Silness, 1963.

I. Oral Mucosal Changes

Epithelial desquamation: It was absent at baseline and after 2 months of using Alcohol and Alcohol free rinses.

Ulcerations: It was absent at baseline and after 2 months of using Alcohol and Alcohol free mouth rinses.

Petechiae: It was absent at baseline and after 2 months of using Alcohol and Alcohol free mouth rinses.

II. Cytological changes

Biosafety analysis of two alcohol (Listerine and Eludril) and alcohol free (Rexidine plus and Hexidine) mouth rinses were assessed by percent cell viability using standard endpoints of cytotoxicity viz. Tetrazolium bromide salt MTT Assay, Neutral red uptake (NRU) Assay and Trypan blue dye exclusion (Trypan blue) Assay in primary culture of oral mucosal cells obtained from different human samples that was pooled together and formed three groups. This was done to avoid heterogeneity due to the variable ethnic groups of the volunteers. On selected samples, post treatment DNA damage was also assessed by micronucleus (MN) frequency and chromosomal aberration (CA) frequency done using Micronucleus Assay and Chromosomal Aberration Assay, respectively.

1. % cell viability

1. MTT Assay

The post treatment MTT Assay percent cell viability of mouth rinses over the periods (24 hr, 48 hr, 72 hr and 96 hr) is summarised in **Table 1 and Fig. 1**. After treatment, percent mean cell viability showed marked decrease in all groups except Hexidine. The decrease in percent mean cell viability was highest in Listerine followed by Eludril, Rexidine plus and Hexidine (Hexidine<Rexidine plus <Eludril< Listerine). In other words, the loss in percent mean cell viability is higher in both the alcohol containing mouth rinses (Listerine and Eludril) than the alcohol free mouth rinses (Rexidine plus and Hexidine).

Table 1: Percent cell viability (Mean ± SE, n=3) of four groups over the periods using MTT Assay

Groups	24 hr	48 hr	72 hr	96 hr
Listerine	77.00 ± 1.73	87.33 ± 2.73	92.33 ± 1.45	96.33 ± 0.67
Eludril	79.67 ± 3.48	85.00 ± 2.31	92.00 ± 1.73	96.67 ± 1.20
Rexidine plus	91.00 ± 2.89	91.00 ± 1.73	96.00 ± 2.52	98.00 ± 1.00
Hexidine	103.00 ± 1.15	99.00 ± 1.15	98.33 ± 0.67	98.33 ± 0.67

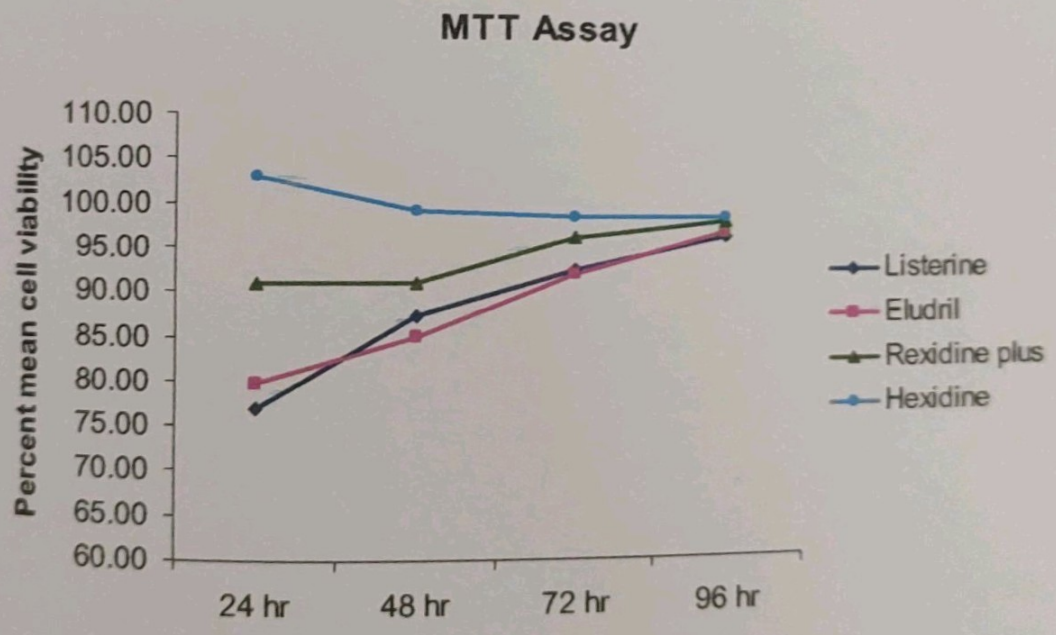
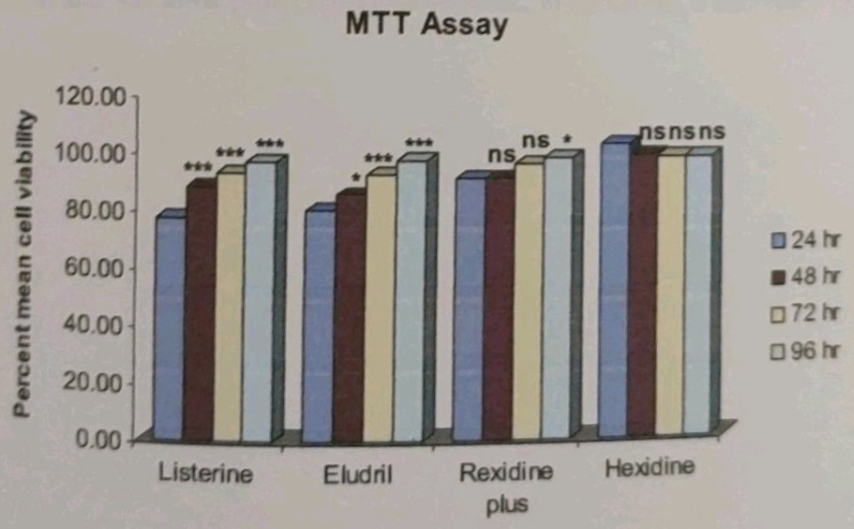


Fig. 1.Percent mean cell viability of four groups over the periods using MTT Assay.

For each group, comparing the difference in percent mean cell viability between periods, Newman-Keuls test showed significant ($p<0.05$ or $p<0.001$) increase in percent cell viability at 48 hr, 72 hr and 96 hr as compared to 24 hr in both the alcohol containing mouth rinse (Listerine and Eludril) **Table 2 and Fig. 2.** In both the alcohol containing mouth rinses, it also increased significantly ($p<0.01$ or $p<0.001$) at 96 hr as compared to 48 hr. Moreover, in Eludril, it also increased significantly ($p<0.05$) at 72 hr as compared to 48 hr. In non-alcoholic mouth rinses,Rexidine plus, it increased significantly ($p<0.05$) at 96 hr as compared to both 24 hr and 48 hr but in Hexidine, it did not differ ($p>0.05$) between the periods i.e. found to be statistically the same.

Table 2: For each group, comparisons (p value) of difference in percent mean cell viability between the periods by Newman-Keuls test

Comparison	Listerine	Eludril	Rexidine plus	Hexidine
24 hr vs. 48 hr	<0.001	0.017	1.000	0.065
24 hr vs. 72 hr	<0.001	<0.001	0.145	0.137
24 hr vs. 96 hr	<0.001	<0.001	0.043	0.082
48 hr vs. 72 hr	0.145	0.019	0.100	0.945
48 hr vs. 96 hr	0.004	<0.001	0.034	0.750
72 hr vs. 96 hr	0.151	0.194	0.769	1.000

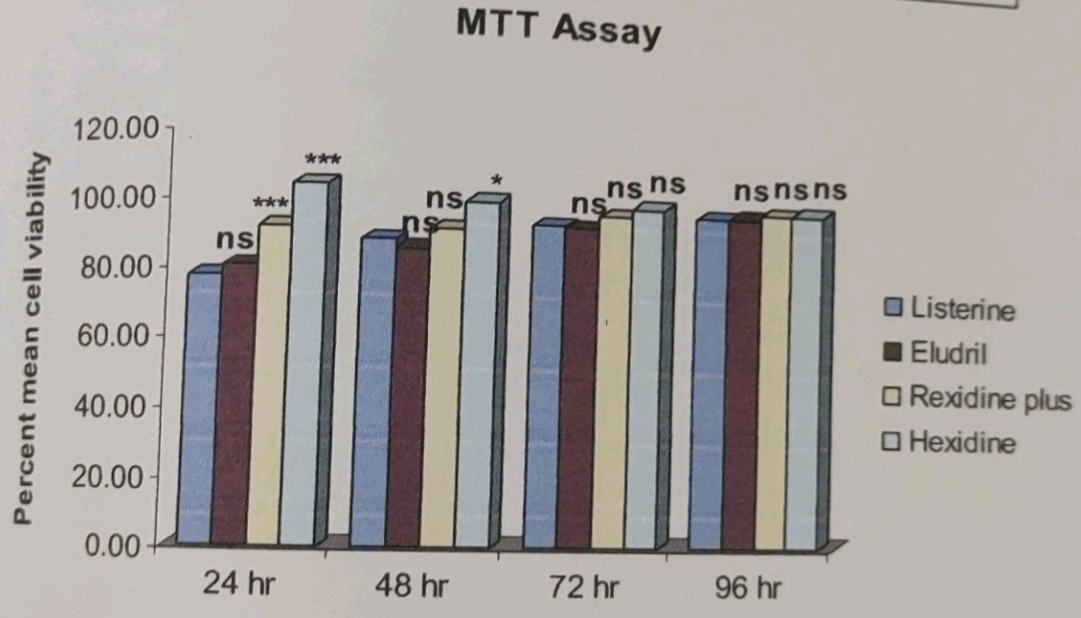


$^{ns}p>0.05$ or $^{*}p<0.05$ or $^{***}p<0.001$- as compared to 24 hr

Fig. 2. For each group, comparisons of difference in percent mean cell viability between the periods using MTT Assay.

Table 3: For each period, comparisons (p value) of difference in percent mean cell viability between the groups by Newman-Keuls test

Comparison	24 hr	48 hr	72 hr	96 hr
Listerine vs. Eludril	0.329	0.392	0.902	0.902
Listerine vs. Rexidine plus	<0.001	0.373	0.184	0.809
Listerine vs. Hexidine	<0.001	0.011	0.259	0.942
Eludril vs. Rexidine plus	0.002	0.143	0.312	0.623
Eludril vs. Hexidine	<0.001	0.002	0.258	0.923
Rexidine plus vs. Hexidine	0.008	0.139	0.904	0.992



$^{ns}p>0.05$ or $^{*}p<0.05$ or $^{***}p<0.001$- as compared to Listerine

Fig. 3. For each period, comparisons of difference in percent mean cell viability between the groups using MTT Assay.

2. NRU Assay

The post treatment NRU Assay percent cell viability of four groups over the periods (24 hr, 48 hr, 72 hr and 96 hr) is summarised in **Table 4 and Fig. 4**. After treatment, percent mean cell viability in both alcohol containing mouth rinses (Listerine and Eludril) increase linearly with time and the increase was evident higher in Listerine than Eludril. In contrast, in alcohol free mouth rinses (Rexidine plus and Hexidine) it decreased with time and the decrease was evident higher in Hexidine than Rexidine plus.

Table 4: Percent cell viability (Mean ± SE, n=3) of four groups over the periods using NRU Assay

Groups	24 hr	48 hr	72 hr	96 hr
Listerine	81.00 ± 2.31	87.33 ± 1.45	92.67 ± 1.76	100.33 ± 1.33
Eludril	86.67 ± 2.60	91.00 ± 2.31	95.00 ± 1.15	97.00 ± 1.15
Rexidine plus	102.00 ± 2.89	99.00 ± 1.73	97.33 ± 0.67	97.67 ± 1.33
Hexidine	105.00 ± 1.73	101.00 ± 2.31	97.67 ± 0.67	97.67 ± 0.88

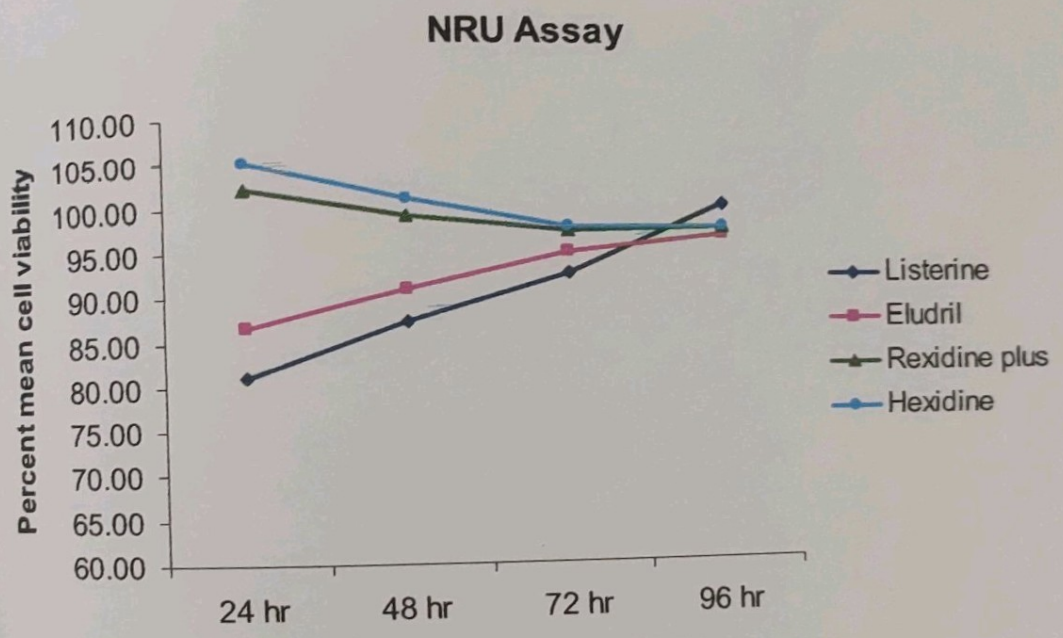
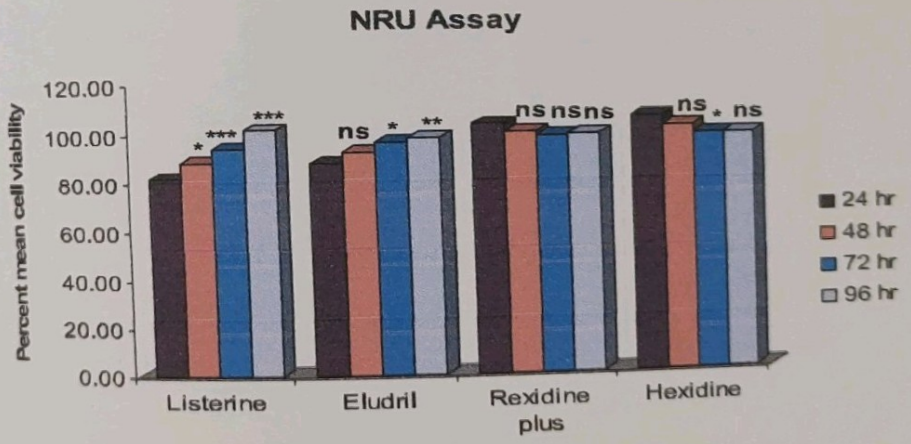


Fig. 4.Percent mean cell viability of four groups over the periods using NRU Assay.

for each group, comparing the difference in percent mean cell viability between periods, Kruskal-Wallis test showed significant ($p<0.05$ or $p<0.001$) increase in percent cell viability at 72 hr and 96 hr as compared to 24 hr in both alcohol containing mouth wash (Listerine and Ehudril) **Table 5 and Fig. 5**.Further, in listerine, it also increase significantly ($p<0.05$ or $p<0.01$) at 48 hr and 96 hr as compared to 24 hr and 48 hr respectively. In contrast, in alcohol free mouth wash chlorhexidine, it decrease significantly ($p<0.05$) at 72 hr as compared to 24 hr but in chlorhexidine plus, it did not differ ($p>0.05$) between the periods i.e. found to be statistically the same.

Table 5: For each group, comparisons (p value) of difference in percent mean cell viability between the periods by Newman-Keuls test

Comparison	Listerine	Eludril	Rexidine plus	Hexidine
24 hr vs. 48 hr	0.031	0.172	0.579	0.220
24 hr vs. 72 hr	<0.001	0.012	0.500	0.044
24 hr vs. 96 hr	<0.001	0.002	0.524	0.057
48 hr vs. 72 hr	0.077	0.220	0.951	0.493
48 hr vs. 96 hr	0.001	0.073	0.939	0.615
72 hr vs. 96 hr	0.063	0.399	0.888	1.000



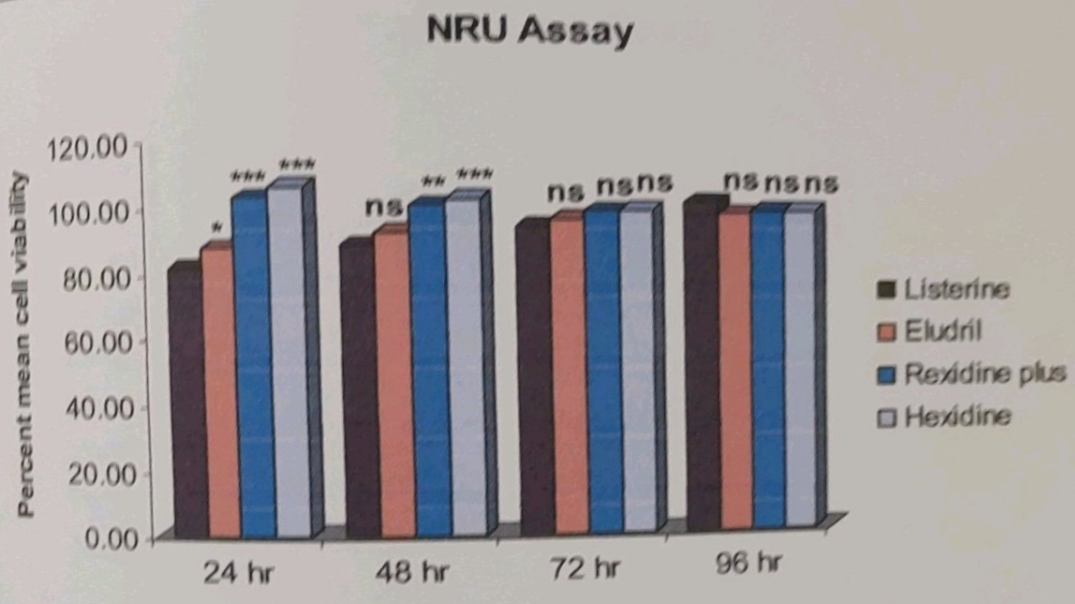
$^{ns}p>0.05$ or $^{*}p<0.05$ or $^{**}p<0.01$ or $^{***}p<0.001$- as compared to 24 hr

Fig. 5. For each group, comparisons of difference in percent mean cell viability between the periods using NRU Assay.

Similarly, for each period, comparing the difference in percent mean cell viability between the groups, Newman-Keuls test showed significantly ($p<0.001$) different and higher percent cell viability in alcohol free mouth rinses (Rexidine plus and Hexidine) as compared to both the alcohol containing mouth rinses (Listerine and Eludril) at 24 hr**Table 6 and Fig. 6**. Further, at 24 hr, the percent mean cell viability of Eludril was also found significantly ($p<0.05$) different and higher as compared to Listerine. At 48 hr, the percent mean cell viability of both alcohol free mouth rinse was found significantly ($p<0.01$ or $p<0.001$) different and higher as compared to alcohol containing mouth rinse. However, at both 72 hr and 96 hr, it did not differ ($p>0.05$) among the groups i.e. found to be statistically the same. However, at final evaluation, the increase in percent mean cell viability (i.e. mean change from 24 hr to 96 hr) of Listerine (19.3%) was higher than Eludril (10.7%). In contrast, the decrease in percent mean cell viability of Hexidine (7.0%) was higher than Rexidine plus (4.2%).

Table 6: For each period, comparisons (p value) of difference in percent mean cell viability between the groups by Newman-Keuls test

Comparison	24 hr	48 hr	72 hr	96 hr
Listerine vs. Eludril	0.031	0.154	0.359	0.832
Listerine vs. Rexidine plus	<0.001	0.002	0.265	0.823
Listerine vs. Hexidine	<0.001	<0.001	0.438	0.713
Eludril vs. Rexidine plus	<0.001	0.069	0.625	0.962
Eludril vs. Hexidine	<0.001	0.014	0.892	0.993
Rexidine plus vs. Hexidine	0.241	0.707	0.999	1.000



$^{ns}p>0.05$ or $^{*}p<0.05$ or $^{**}p<0.01$ or $^{***}p<0.001$- as compared to Listerine

Fig. 6. For each period, comparisons of difference in percent mean cell viability between the groups using NRU Assay.

3. Trypan blue Assay

The post treatment Trypan blueAssay, percent cell viability of four groups over the periods is summarised in **Table 7 and Fig. 7**. It shows that the percent mean cell viability in all four groups increase linearly with time and the increase was evident highest in Rexidine plus followed by Hexidine, Listerine and Eludril (Eludril< Listerine <Hexidine<Rexidine plus). In other words, loss of percent cell viability were higher in the cells isolated from the buccal cavity of human samples using alcohol free mouth rinses than the samples using alcohol containing mouth rinses.

Table 7: Percent cell viability (Mean ± SE, n=3) of four groups over the periods using Trypan blue Assay

Groups	24 hr	48 hr	72 hr	96 hr
Listerine	94.00 ± 0.87	95.83 ± 0.72	97.23 ± 0.51	98.67 ± 0.55
Eludril	92.75 ± 1.30	95.17 ± 0.82	95.67 ± 0.68	97.16 ± 0.68
Rexidine plus	97.00 ± 1.88	100.75 ± 2.22	101.42 ± 2.49	102.42 ± 2.53
Hexidine	96.00 ± 1.59	98.42 ± 0.68	101.17 ± 0.46	101.17 ± 0.46

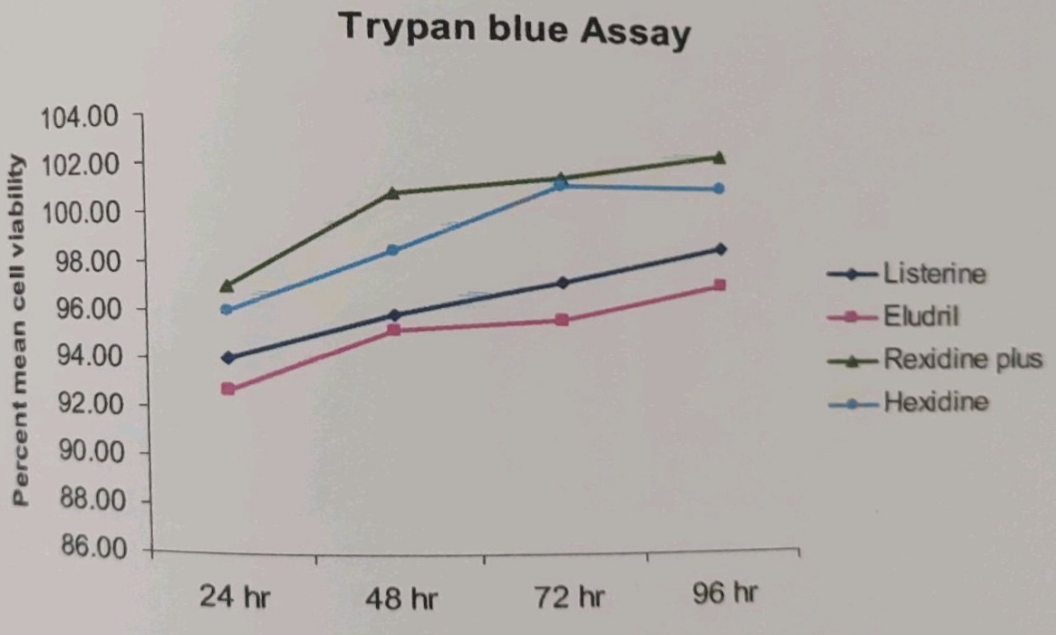


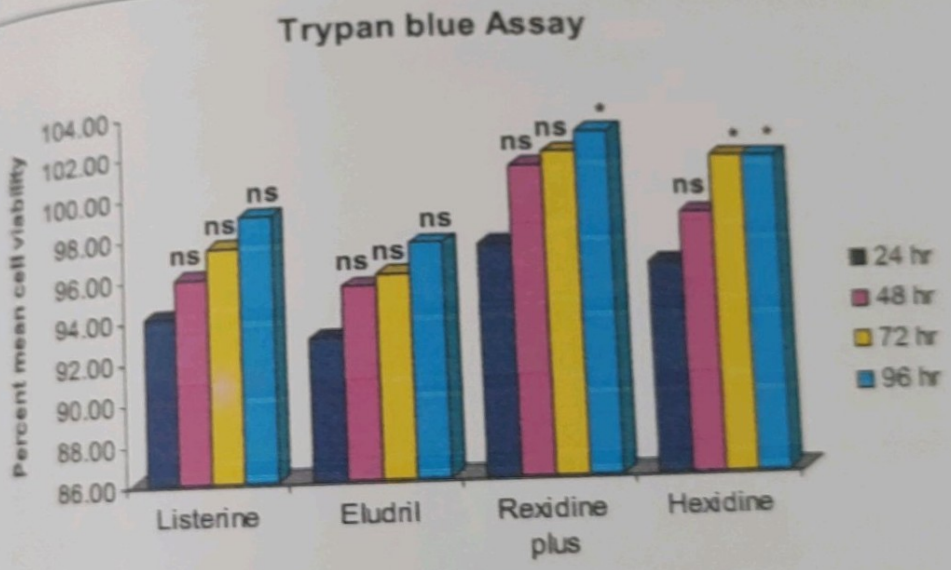
Fig. 7.Percent mean cell viability of four groups over the periods using Trypan blue Assay.

For each group, comparing the difference in percent mean cell viability between periods, Newman-Keuls test showed insignificant ($p>0.05$) difference in percent cell viability between periods in both alcohol containing mouth rinse (Listerine and Eludril) **Table 8 and Fig. 8**

In contrast, in both the alcohol free mouth rinse (Rexidine plus and Hexidine), it increases significantly ($p<0.05$) at 96 hr as compared to 24 hr. Further, in Hexidine, it also increase significantly ($p<0.05$) at 72 hr as compared to 24 hr.

Table 8: For each group, comparisons (p value) of difference in percent mean cell viability between the periods by Newman-Keuls test

Comparison	Listerine	Eludril	Rexidine plus	Hexidine
24 hr vs. 48 hr	0.586	0.229	0.130	0.458
24 hr vs. 72 hr	0.355	0.201	0.095	0.031
24 hr vs. 96 hr	0.078	0.081	0.025	0.026
48 hr vs. 72 hr	0.862	0.730	0.966	0.332
48 hr vs. 96 hr	0.447	0.730	0.770	0.245
72 hr vs. 96 hr	0.578	0.832	0.491	1.000



"p>0.05 or *p<0.05- as compared to 24 hr

Fig. 8. For each group, comparisons of difference in percent mean cell viability between the periods using Trypan blue Assay.

Similarly, for each period, comparing the difference in percent mean cell viability between the groups, Newman-Keuls test showed similar ($p>0.05$) percent cell viability between the groups at all periods i.e. did not differ significantly **Table 9 and Fig. 9**. However, at final evaluation, the increase in percent mean cell viability (i.e. mean change from 24 hr to 96 hr) of Rexidine plus was the highest (5.3%) followed by Hexidine (5.1%), Listerine (4.7%) and Eludril the least (4.5%).

Table 9: For each period, comparisons (p value) of difference in percent mean cell viability between the groups by Newman-Keuls test

Comparison	24 hr	48 hr	72 hr	96 hr
Listerine vs. Eludril	0.523	0.938	0.962	0.861
Listerine vs. Rexidine plus	0.632	0.227	0.348	0.403
Listerine vs. Hexidine	0.834	0.756	0.351	0.411
Eludril vs. Rexidine plus	0.333	0.169	0.183	0.197
Eludril vs. Hexidine	0.553	0.693	0.205	0.334
Rexidine plus vs. Hexidine	0.609	0.459	0.898	0.914

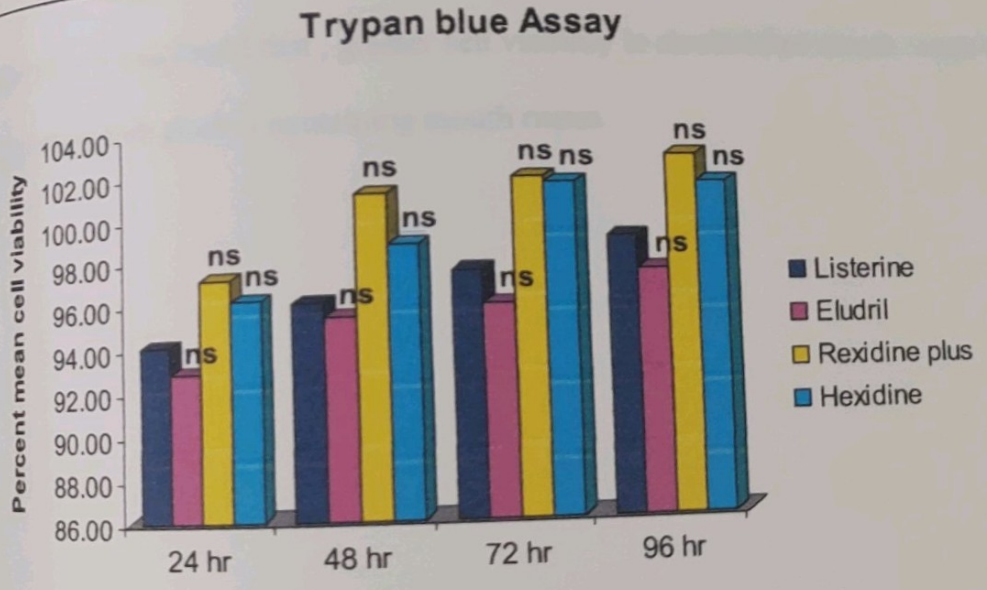
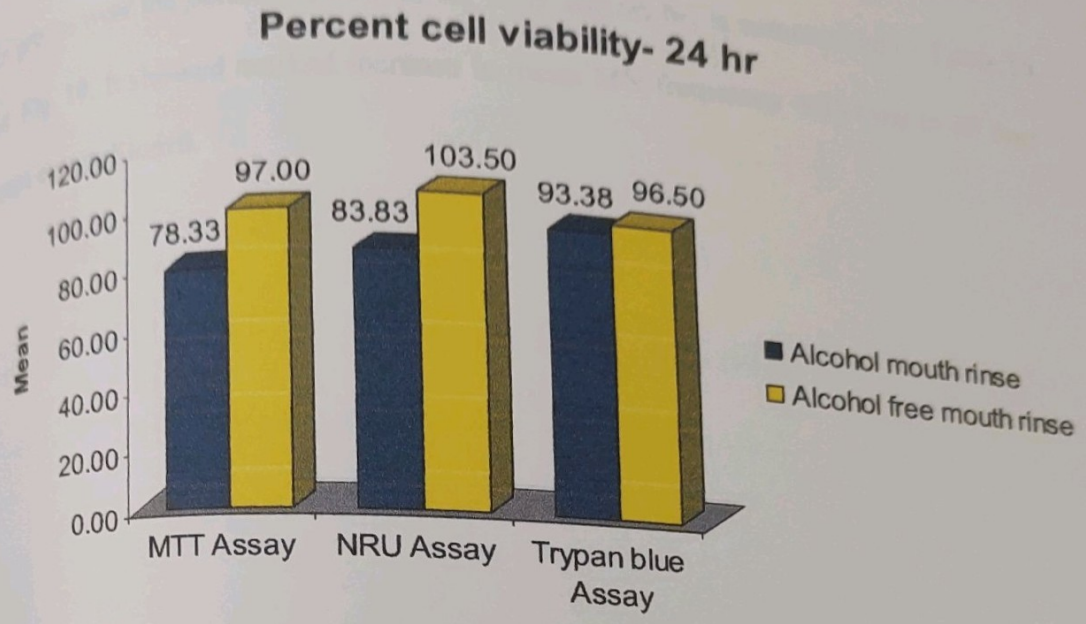


Fig. 9. For each period, comparisons of difference in percent mean cell viability between the groups using **TRYPAN BLUE Assay.**

Comparison of percent cell viability at 24 hr between alcohol containing mouth rinses and alcohol free mouthrinses assessed by using MTT Assay, NRU Assay and Trypan Blue Assay



After 24 hr it was found that , greater cell viability in alcohol free mouth rinses in comparison with alcohol containing mouth rinses.

DNA damage

Micronucleus Assay

The Micronucleus Assay was done to assess the micronucleus (MN) frequency i.e. accumulation of genetic damage in the cells. The post treatment MN frequency of four groups over the periods (24 hr, 48 hr, 72 hr and 96 hr) is summarised in **Table 10** and **Fig. 10**. It showed marked increase in mean MN frequency with time in all four groups except Eludril.

Table 10. MN frequency (Mean ± SE, n=3) of four groups over the periods using Micronucleus Assay

Groups	24 hr	48 hr	72 hr	96 hr
Listerine	3.00 ± 0.58	4.67 ± 0.33	5.00 ± 1.15	3.33 ± 0.88
Eludril	4.33 ± 1.20	4.33 ± 0.33	4.33 ± 0.88	3.33 ± 0.67
Rexidine plus	3.33 ± 0.88	3.33 ± 0.88	3.00 ± 0.58	5.00 ± 1.00
Hexidine	2.67 ± 0.67	3.33 ± 0.33	3.00 ± 1.00	4.33 ± 0.88

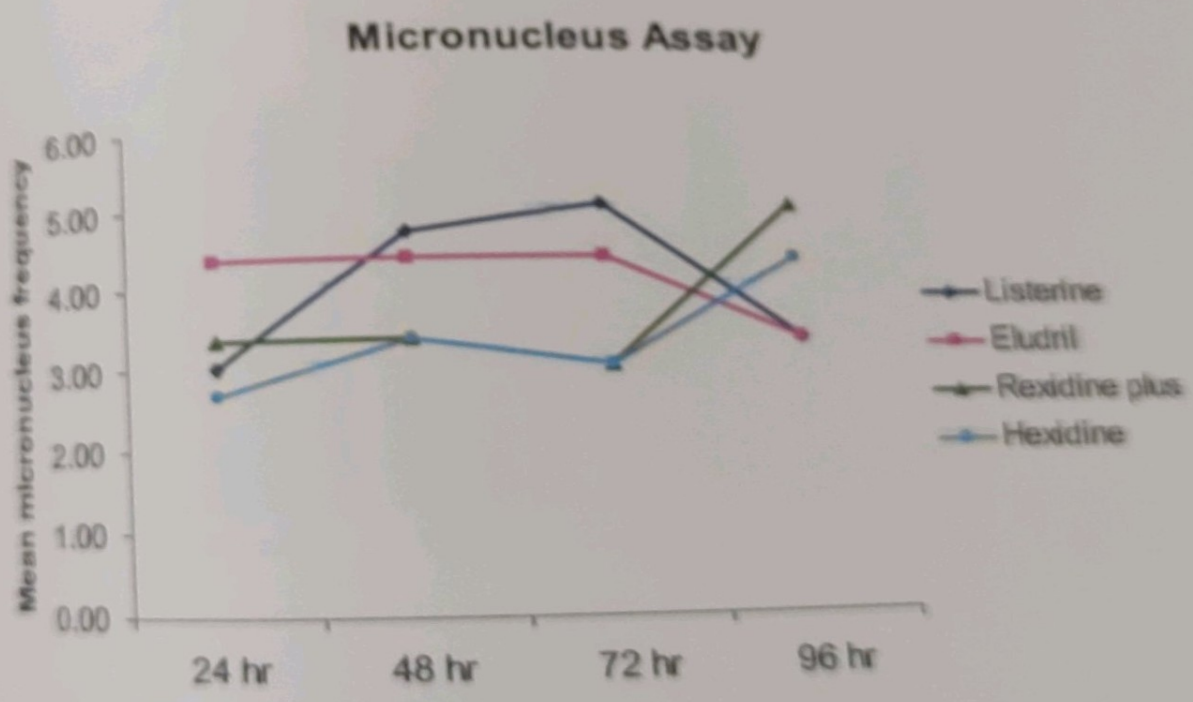


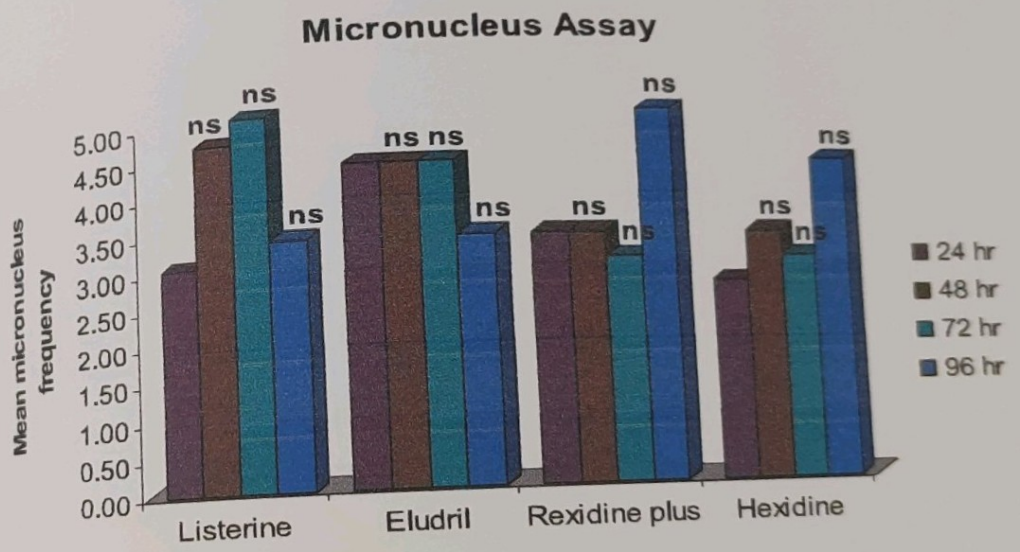
Fig. 10. Mean MN frequency of four groups over the periods using Micronuc

Assay.

each group, comparing the difference in mean MN frequency between the
iods. Newman-Keuls test showed similar ($p>0.05$) MN frequency between periods
ll groups i.e. did not differ significantly **Table 11 and Fig. 11.**

ble 11: For each group, comparisons (p value) of difference in mean MN equency between the periods by Newman-Keuls test

omparison	Listerine	Eludril	Rexidine plus	Hexidine
hr vs. 48 hr	0.945	1.000	1.000	0.993
hr vs. 72 hr	0.876	1.000	1.000	0.960
hr vs. 96 hr	0.787	0.921	0.862	0.945
8 hr vs. 72 hr	0.787	1.000	1.000	0.993
8 hr vs. 96 hr	0.981	0.694	0.925	0.961
2 hr vs. 96 hr	0.945	0.961	0.931	0.969



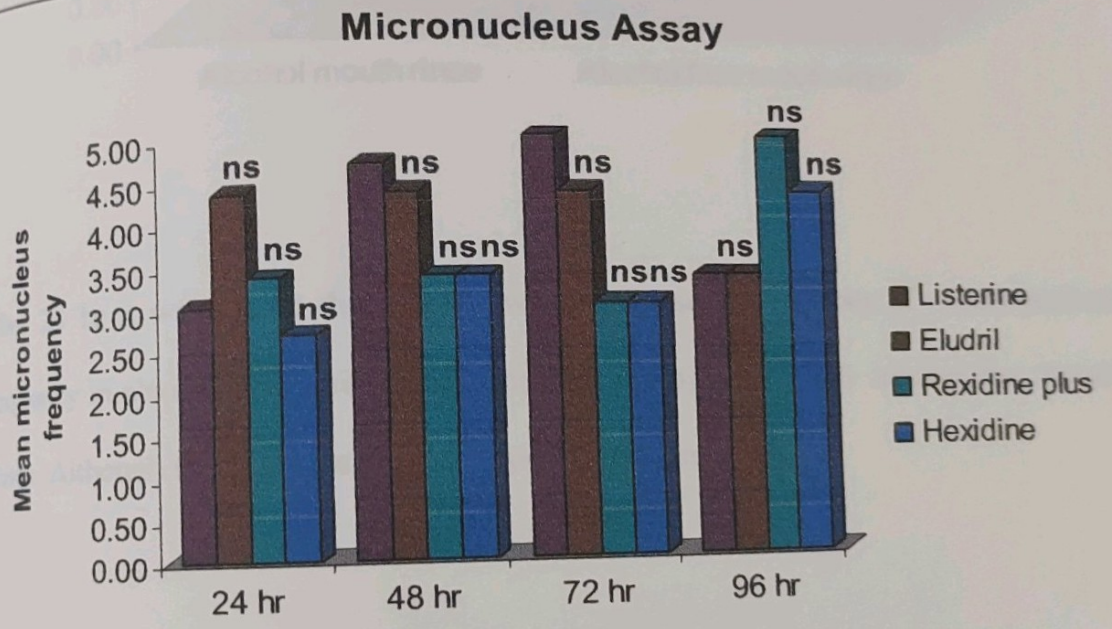
[ns]p>0.05- as compared to 24 hr

Fig. 11. For each group, comparisons of difference in mean MN frequency between the periods using Micronucleus Assay.

Similarly, for each period, comparing the difference in mean MN frequency between the groups, Newman-Keuls test showed similar ($p>0.05$) MN frequency between groups at all periods i.e. also not differ significantly **Table 12 and Fig. 12**. However, at final evaluation, the increase in mean MN frequency (i.e. mean change from 24 hr to 96 hr) of Hexidine was found to be the highest (38.5%) followed by Rexidine plus (33.3%) and Listerine (10.0%). In contrast, in Eludril, it decreased by 23.1%.

Table 12: For each period, comparisons (p value) of difference in percent mean cell viability between the groups by Newman-Keuls test

Comparison	24 hr	48 hr	72 hr	96 hr
Listerine vs. Eludril	0.959	0.998	0.832	1.000
Listerine vs. Rexidine plus	1.000	0.937	0.885	0.943
Listerine vs. Hexidine	0.991	0.959	0.862	0.974
Eludril vs. Rexidine plus	0.820	0.820	0.988	0.869
Eludril vs. Hexidine	0.943	0.906	0.982	0.820
Rexidine plus vs. Hexidine	1.000	1.000	1.000	0.992

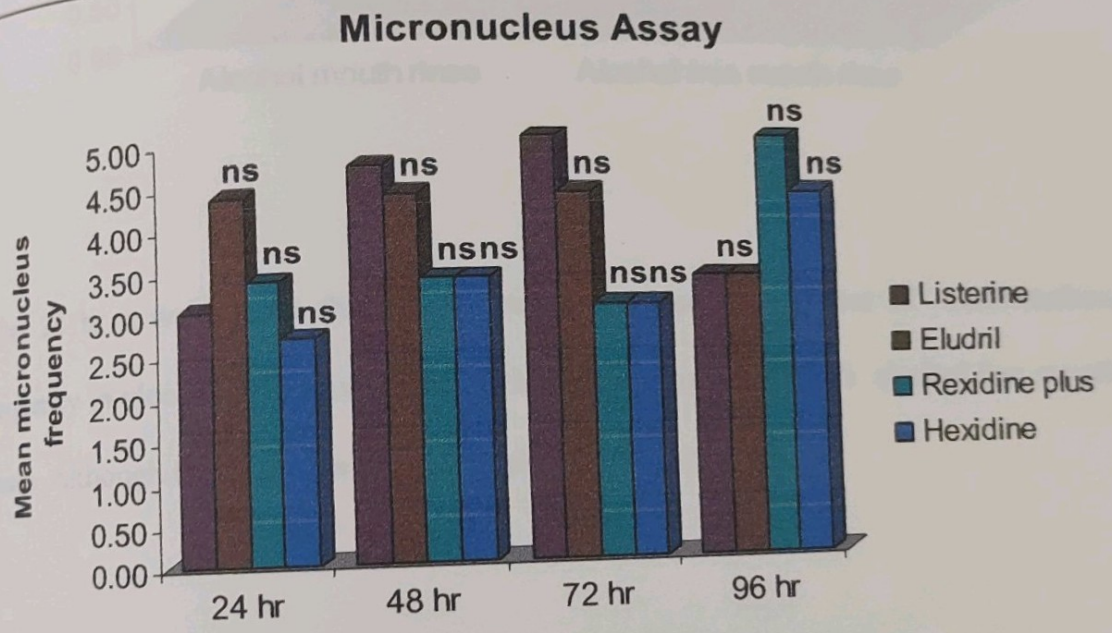


[ns]p>0.05- as compared to Listerine

Fig. 12. For each period, comparisons of difference in mean MN frequency between the groups using Micronucleus Assay.

Table 12: For each period, comparisons (p value) of difference in percent mean cell viability between the groups by Newman-Keuls test

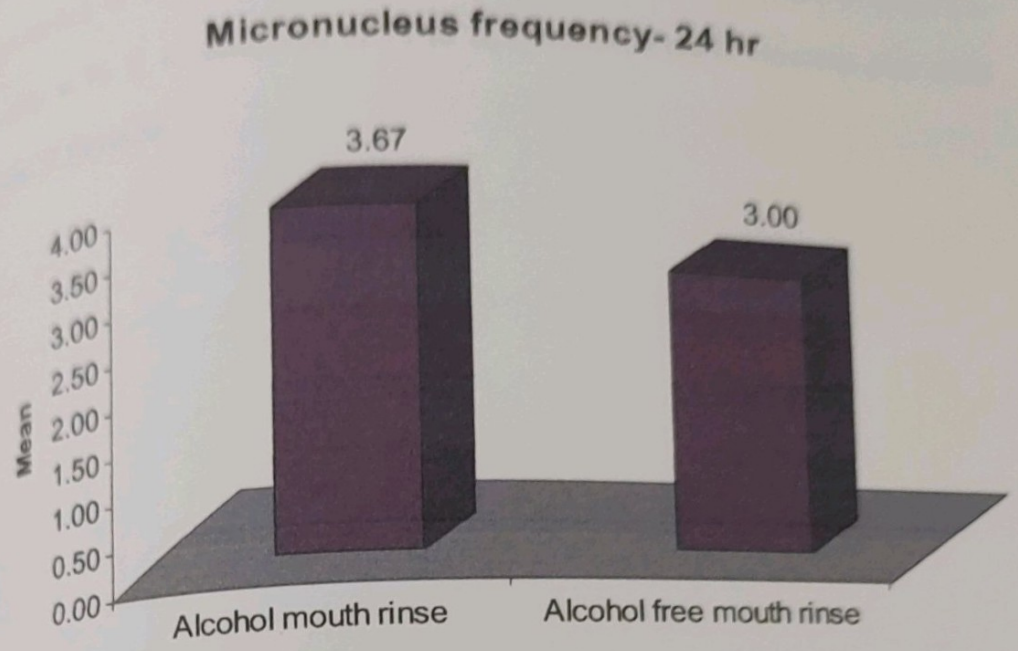
Comparison	24 hr	48 hr	72 hr	96 hr
Listerine vs. Eludril	0.959	0.998	0.832	1.000
Listerine vs. Rexidine plus	1.000	0.937	0.885	0.943
Listerine vs. Hexidine	0.991	0.959	0.862	0.974
Eludril vs. Rexidine plus	0.820	0.820	0.988	0.869
Eludril vs. Hexidine	0.943	0.906	0.982	0.820
Rexidine plus vs. Hexidine	1.000	1.000	1.000	0.992



ns p>0.05- as compared to Listerine

Fig. 12. For each period, comparisons of difference in mean MN frequency between the groups using Micronucleus Assay.

Comparison of micronucleus frequency at 24 hr between alcohol containing mouth rinses and alcohol free mouthrinses assessed by using Micronucleus Assay



After 24 hr it was found that , there was increase in number of micronucleus frequency in alcohol containing mouth rinses in comparison with alcohol free mouth rinses .Although this increase was biologically insignificant.

Chromosomal Aberration Assay

Chromosomal Aberration Assay was also done to assess the chromosomal erration (CA) frequency i.e. accumulation of genetic damage in the cells. The post atment CA frequency of four groups over the periods (24 hr, 48 hr, 72 hr and 96 hr) summarised in **Fig. 13 and Table 13** . It showed marked increase in mean CA equency at 96 hr as compared to 24 hr in Listerine, Eludril, Rexidine plus while crease in Hexidine.

Table 13: CA frequency (Mean ± SE, n=3) of four groups over the periods using Micronucleus Assay

Groups	24 hr	48 hr	72 hr	96 hr
Listerine	1.33 ± 0.33	1.67 ± 0.33	1.67 ± 0.33	2.67 ± 0.67
Eludril	1.33 ± 0.33	1.33 ± 0.33	1.67 ± 0.33	1.67 ± 0.33
Rexidine plus	1.33 ± 0.33	1.33 ± 0.33	2.67 ± 0.33	1.67 ± 0.67
Hexidine	1.67 ± 0.33	1.67 ± 0.67	1.67 ± 0.33	1.33 ± 0.33

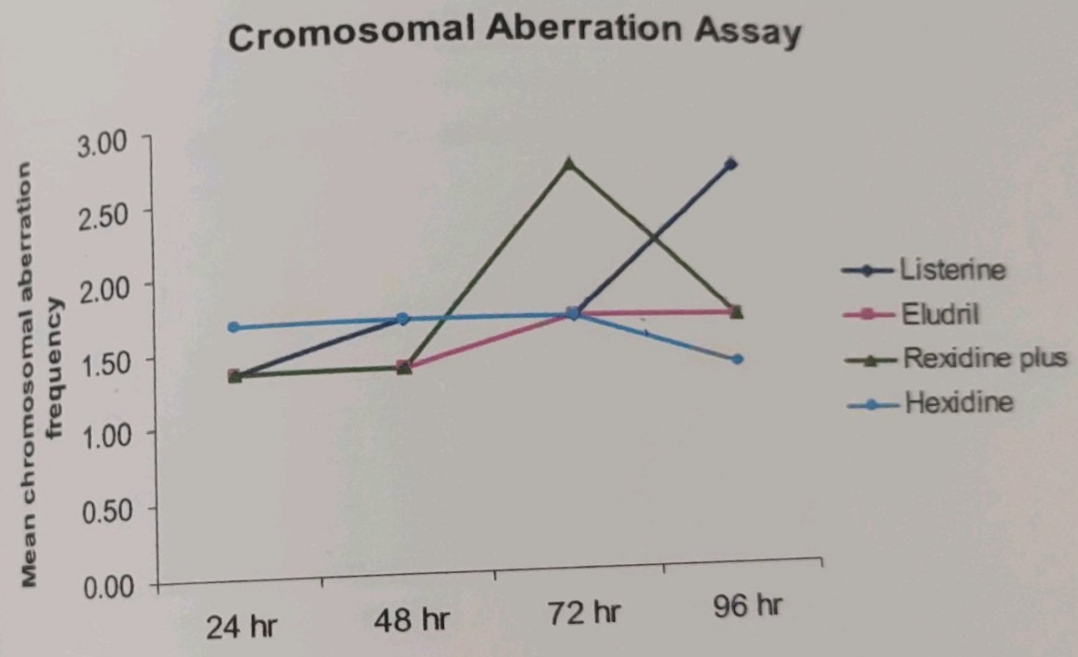


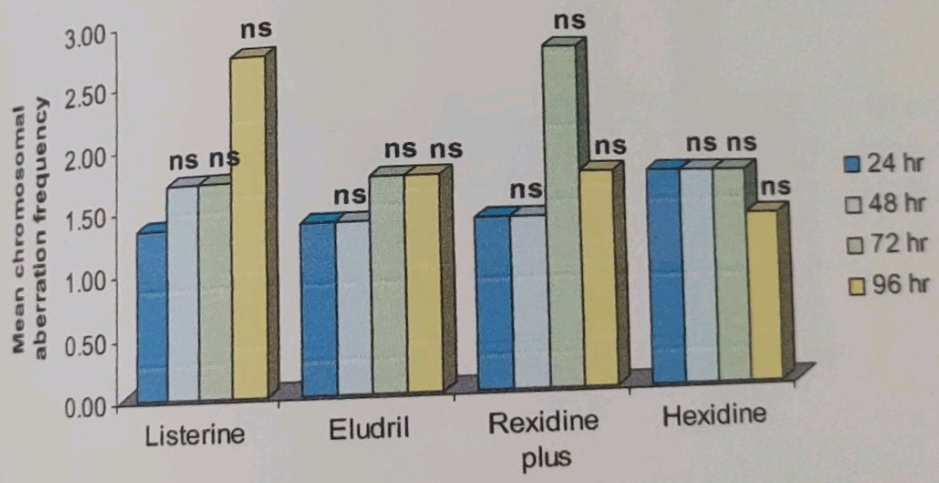
Fig. 13. Mean CA frequency of four groups over the periods using Micronucleus Assay.

For each group, comparing the difference in mean CA frequency between the periods, Newman-Keuls test showed similar ($p>0.05$) CA frequency between periods in all groups i.e. did not differ significantly **Table 14 and Fig. 14.**

Table 14: **For each group, comparisons (p value) of difference in mean CA frequency between the periods by Newman-Keuls test**

Comparison	Listerine	Eludril	Rexidine plus	Hexidine
24 hr vs. 48 hr	1.000	1.000	1.000	1.000
24 hr vs. 72 hr	0.999	0.984	0.655	1.000
24 hr vs. 96 hr	0.741	1.000	1.000	0.998
48 hr vs. 72 hr	1.000	0.995	0.687	1.000
48 hr vs. 96 hr	0.406	1.000	1.000	0.602
72 hr vs. 96 hr	0.804	1.000	0.521	0.984

Chromosomal Aberration Assay



"p>0.05- as compared to 24 hr

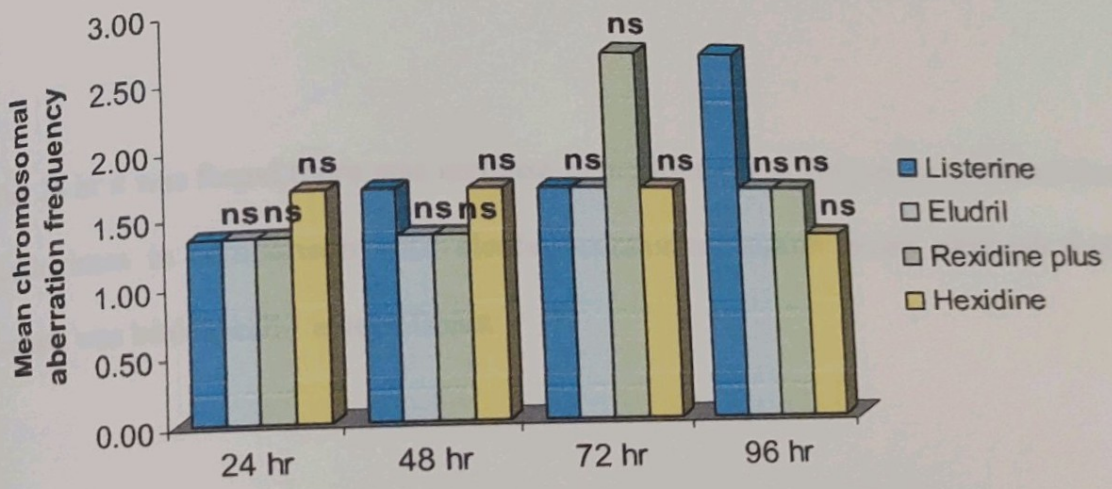
Fig. 14. For each group, comparisons of difference in mean CA frequency between the periods using Micronucleus Assay.

similarly, for each period, comparing the difference in mean CA frequency between e groups, Newman-Keuls test showed similar ($p>0.05$) CA frequency between roups at all periods i.e. also not differ significantly **Table 15 and Fig. 15**. However, final evaluation, the increase in mean CA frequency (i.e. mean change from 24 hr 96 hr) of Listerine was found highest (50.0%) followed by both Eludril and exidine plus (20.0%). In contrast, in Hexidine, it decreased by 20.0%.

Table 15: For each period, comparisons (p value) of difference in percent mean cell viability between the groups by Newman-Keuls test

Comparison	24 hr	48 hr	72 hr	96 hr
Listerine vs. Eludril	1.000	1.000	1.000	0.223
Listerine vs. Rexidine plus	1.000	1.000	0.689	0.544
Listerine vs. Hexidine	1.000	1.000	1.000	0.482
Eludril vs. Rexidine plus	1.000	1.000	0.623	1.000
Eludril vs. Hexidine	0.999	0.942	1.000	1.000
Rexidine plus vs. Hexidine	1.000	0.993	0.544	0.993

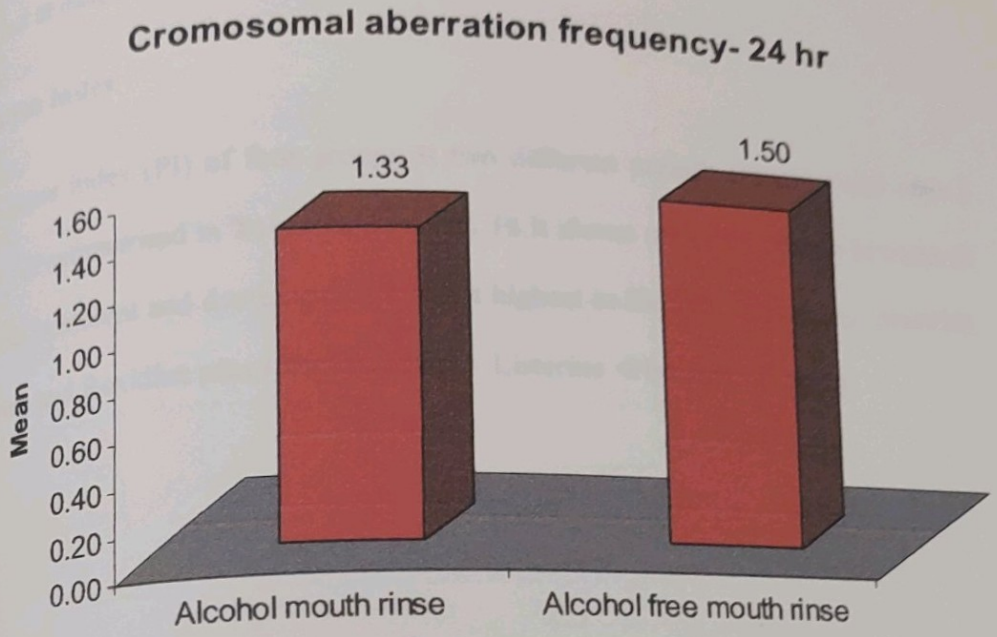
Chromosomal Aberration Assay



[ns]p>0.05- as compared to Listerine

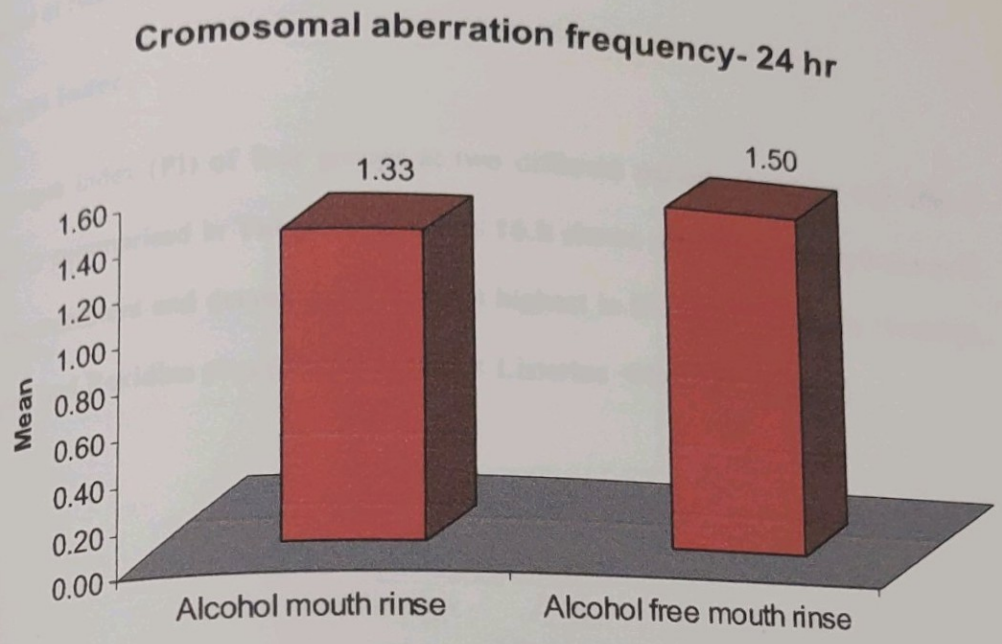
Fig. 15. For each period, comparisons of difference in mean CA frequency between the groups using Micronucleus Assay

Comparison of chromosomal aberration frequency at 24 hr between alcohol containing mouth rinses and alcohol free mouthrinses assessed by using Chromosomal Aberration Assay.



After 24 hr it was found,there was increase in number of CA frequency in alcohol free mouth rinses in comparison with alcohol containing mouth rinses .although these increase was biologically insignificant.

Comparison of chromosomal aberration frequency at 24 hr between alcohol containing mouth rinses and alcohol free mouthrinses assessed by using Chromosomal Aberration Assay.



After 24 hr it was found,there was increase in number of CA frequency in alcohol free mouth rinses in comparison with alcohol containing mouth rinses .although these increase was biologically insignificant.

Clinical parameters

he effect of alcohol containing mouth rinses i.e Listerine and Eludril and alcohol
ee i.eRexidine plus and Hexidine mouth rinses were also assessed on clinical
arameters viz. plaque index (PI) and gingival index (GI). For this, PI and GI were
ssessed at baseline and after 2 month .

Plaque Index

The plaque index (PI) of four groups at two different periods (baseline and after 2 month) is summarised in **Table 16 and Fig. 16**.It shows marked decrease in mean PI after the treatment and decrease was evident highest in Eludril followed by Hexidine, Listerine and Rexidine plus (Rexidine plus < Listerine <Hexidine<Eludril).

Table 16: **PI (Mean ± SE, n=30) of four groups at two different periods and comparison (p value) of difference in mean PI.**

Groups	Baseline	2 month	Comparison
Listerine	0.83 ± 0.11	0.40 ± 0.09	<0.001
Eludril	0.90 ± 0.12	0.30 ± 0.09	<0.001
Rexidine plus	0.87 ± 0.10	0.43 ± 0.10	<0.001
Hexidine	0.90 ± 0.09	0.37 ± 0.09	<0.001

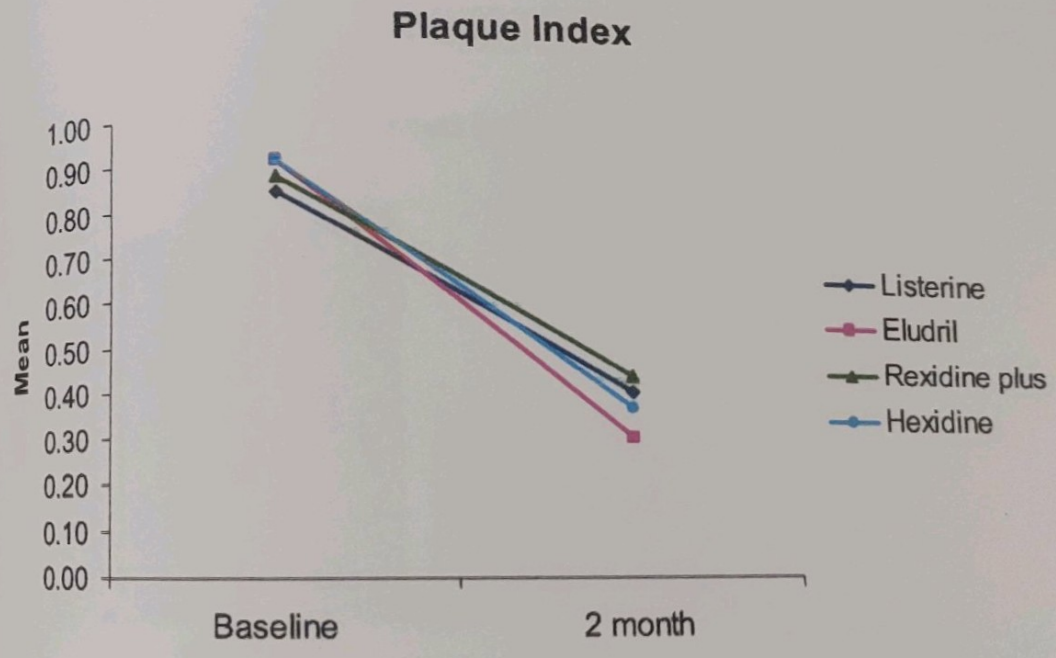
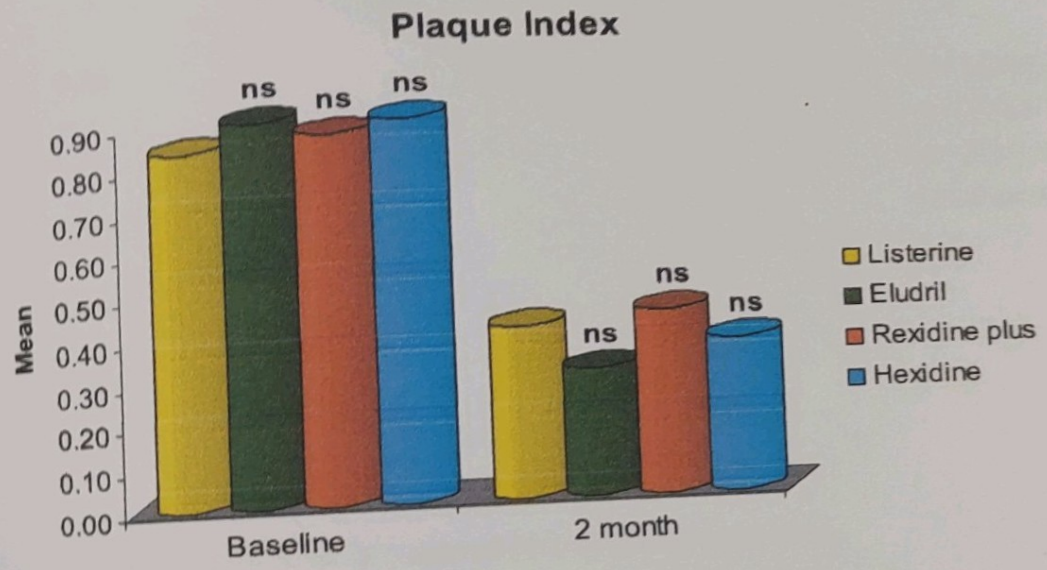


Fig. 16. Mean PI of four groups at two different periods.

Similarly, for each period, comparing the difference in mean PI between the groups, Newman-Keuls test showed insignificant ($p>0.05$) difference in PI between groups at both periods (baseline and 2 month) i.e. did not differ significantly **Table 18 and Fig. 18**. However, at final evaluation, the decrease in mean PI (i.e. mean change from baseline to 2 month) of Eludril was the highest (66.7%) followed by Hexidine (59.3%), Listerine (52.0%) and Rexidine plus the least (50.0%).

Table 18: For each period, comparisons (p value) of difference in mean PI between the groups by Newman-Keuls test

Comparison	Baseline	2 month
Listerine vs. Eludril	0.965	0.757
Listerine vs. Rexidine plus	0.813	0.813
Listerine vs. Hexidine	0.884	0.813
Eludril vs. Rexidine plus	0.970	0.779
Eludril vs. Hexidine	1.000	0.636
Rexidine plus vs. Hexidine	0.813	0.884



^{ns}p>0.05- as compared to Listerine

Fig. 18. For each period, comparisons of difference in mean PI between the groups.

2. Gingival Index

The gingival index (GI) of four groups at two different periods (baseline and after 2 month) is summarised in **Table 19 and Fig. 19.**.Like PI, there was marked decrease in mean GI after 2 months and the decrease was evident highest in Eludril followed by Hexidine, Listerine and Rexidine plus (Rexidine plus < Listerine <Hexidine<Eludril).

Table 19: GI (Mean ± SE, n=30) of four groups at two different periods and comparison (p value) of difference in mean GI.

Groups	Baseline	2 month	Comparison
Listerine	0.97 ± 0.10	0.53 ± 0.11	0.001
Eludril	1.03 ± 0.09	0.47 ± 0.10	<0.001
Rexidine plus	1.00 ± 0.13	0.60 ± 0.09	0.001
Hexidine	1.03 ± 0.13	0.53 ± 0.10	<0.001

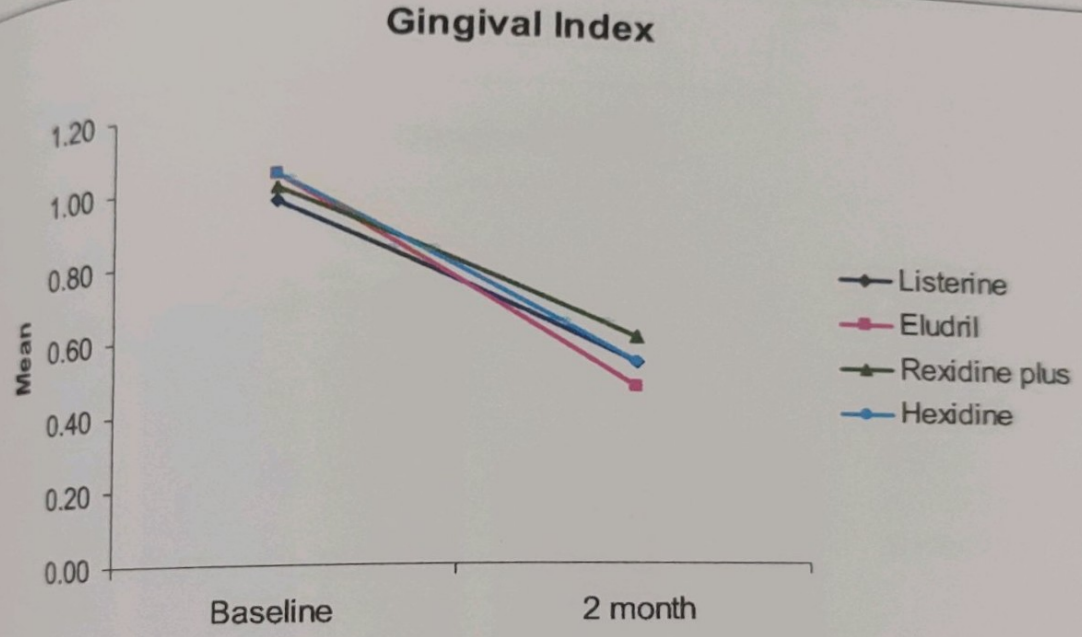


Fig. 19. Mean GI of four groups at two different periods.

Similarly, for each period, comparing the difference in mean GI between the groups, Newman-Keuls test showed insignificant ($p>0.05$) difference in GI between groups at both periods (baseline and 2 month) i.e. did not differ significantly **Table 20 and Fig. 20**. However, at final evaluation, the decrease in mean GI (i.e. mean change from baseline to 2 month) of Eludril was found highest (54.8%) followed by Hexidine (48.4%), Listerine (44.8%) and Rexidine plus the least (40.0%).

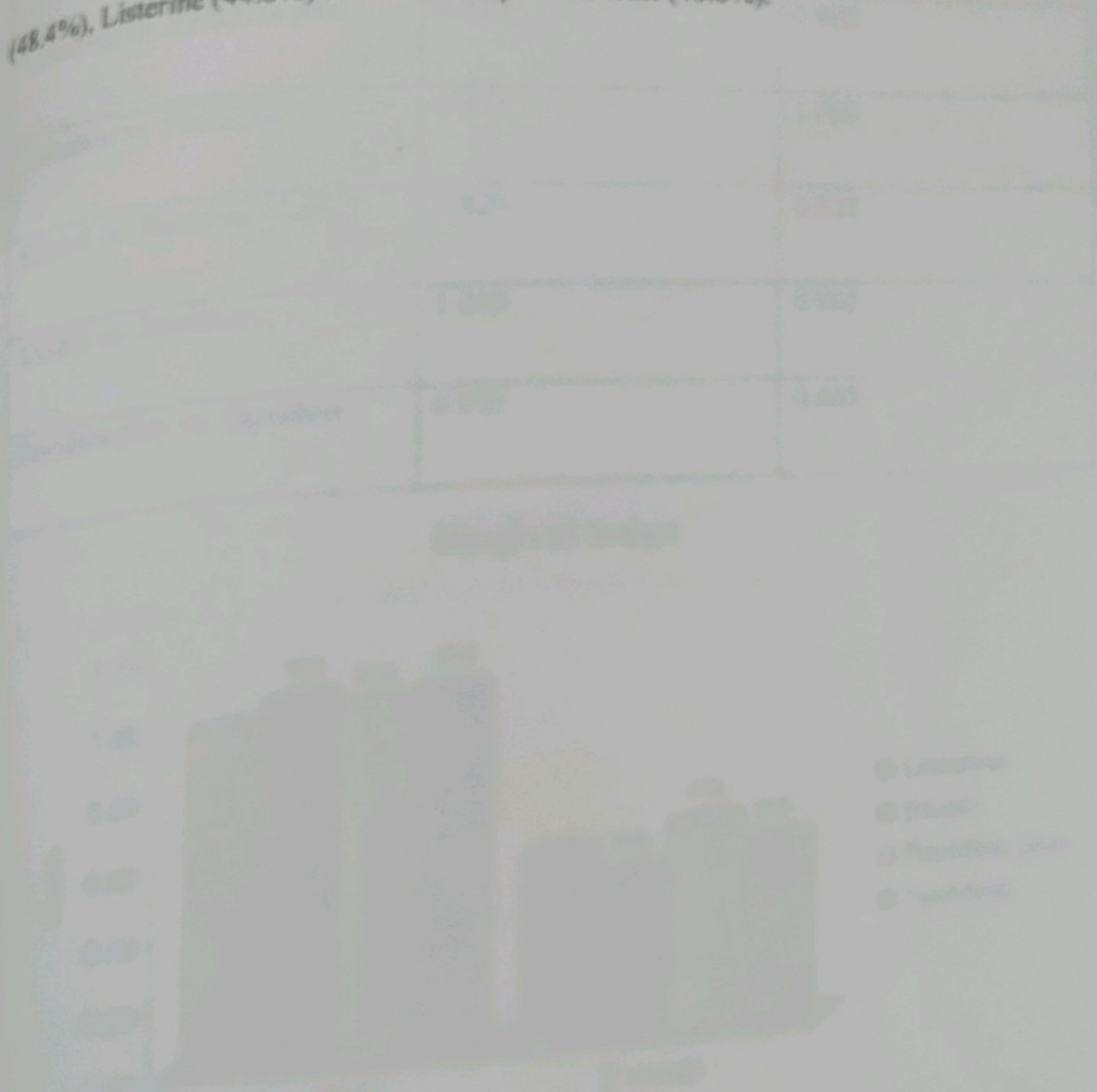
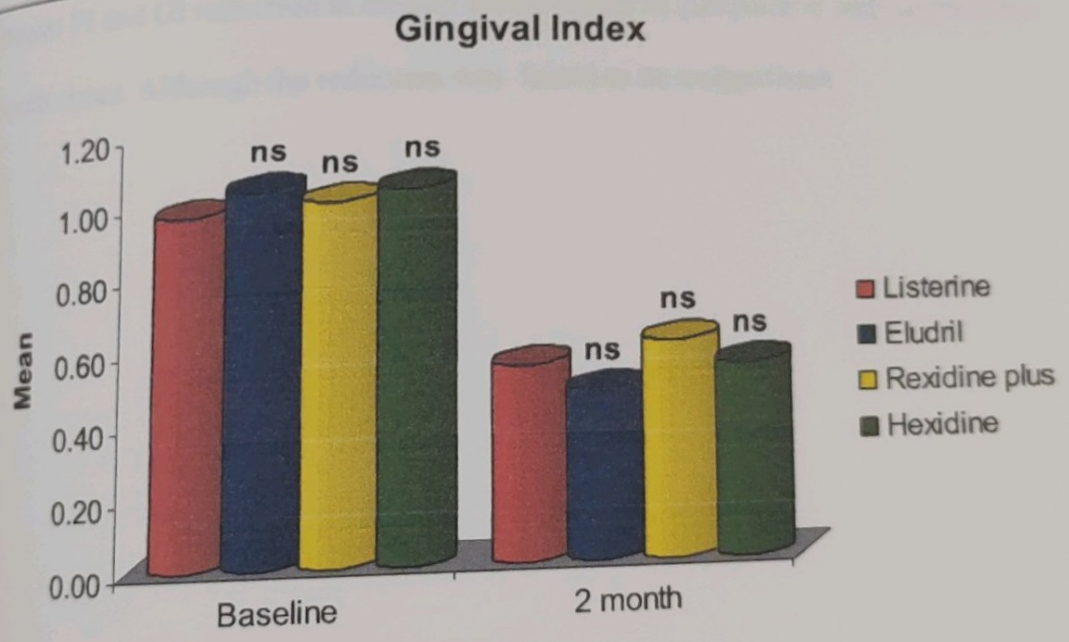


Table 20: For each period, comparisons (p value) of difference in mean GI between the groups by Newman-Keuls test

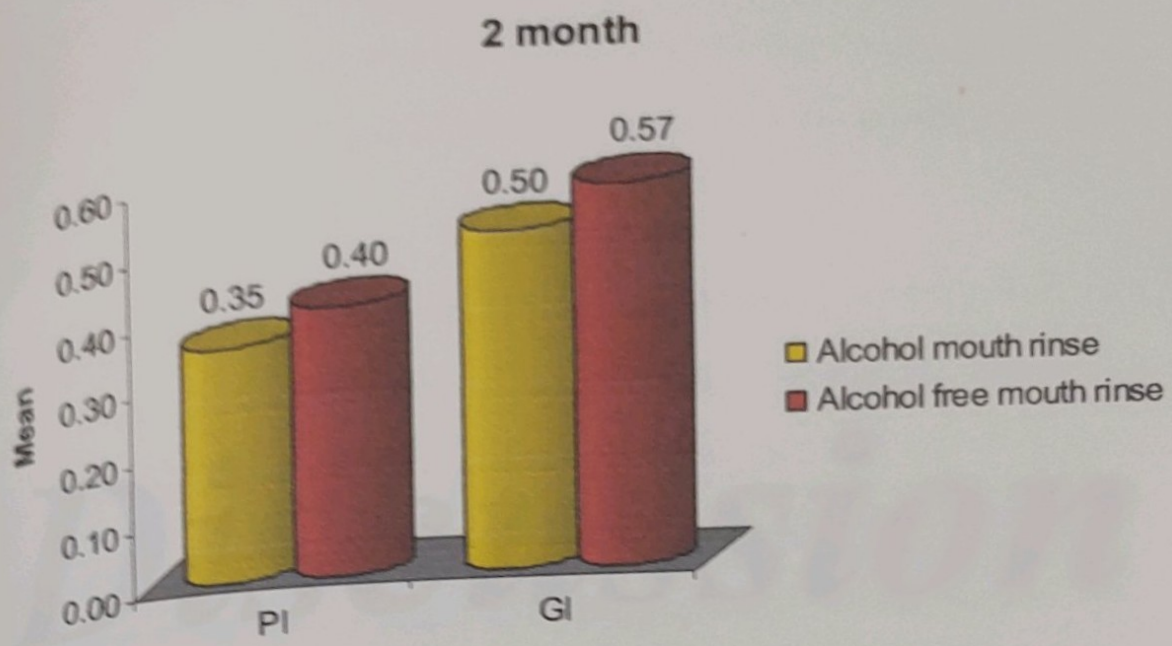
Comparison	Baseline	2 month
Listerine vs. Eludril	0.902	0.665
Listerine vs. Rexidine plus	0.829	0.902
Listerine vs. Hexidine	0.973	1.000
Eludril vs. Rexidine plus	0.829	0.822
Eludril vs. Hexidine	1.000	0.902
Rexidine plus vs. Hexidine	0.975	0.665



[ns]p>0.05- as compared to Listerine

Fig. 20. For each period, comparisons of difference in mean GI between the groups.

Comparison of PI and GI between alcohol mouth rinse and alcohol free mouth rinse after 2 month.



Greater PI and GI reduction in alcohol mouth rinses in comparison with alcohol free mouth rinses. Although the reduction was found to be insignificant.

Discussion

Mouth rinses are used widely worldwide, mainly for their capacity to control dental plaque and gingivitis. Daily use of mouth rinses has been recommended for the prevention of periodontal disease and control of caries.[22, 54] According to Silverman & Wilder , when used in conjunction with brushing and flossing, they are an important method for reducing plaque, gingivitis, and preventing or minimizing periodontal disease.[36]

Many mouth rinses with antiplaque properties contain pharmaceutical-grade denatured alcohol as a vehicle. Concern has been raised regarding the potential for alcohol-containing rinses to cause adverse effects.In fact, the use of alcohol-containing mouth rinses should be restricted in high-risk populations.[43] According to McCullough & Farah, there is sufficient evidence to accept that alcohol-containing mouth washes is suspected to contribute increased risk of developing oral cancer.[34, 55]

Lemos&Villoria stated that the correlation between oral cancer and alcohol-based mouth rinses was small, weak, inconsistent, and even contradictory.[56]

The role of alcohol containing mouth rinses in oral tissues has also been studied and found to be non-cytotoxic and also there was absence of histopathological effects in oral tissues found by Koschier et al.[43] According to Silverman & Wilder , antimicrobial mouth rinses are safe and effective.[36]

Although not therapeutically active, Ethanol in the mouthwash has been proven to produce multiple other effects many of which are not beneficial and un-necessary for the user. These range from a characteristic burning sensation upon contact with the oral mucosa.[57] Due to the astringent action of ethanol, the use of high alcohol mouthwashes in patients with radiation mucositis is not recommended.[48]

Alcohol is an irritant to epithelium, In a study ,areas of hyper-parakeratinazation, loss of cellular cohesion in basal cell layers with mononuclear inflammatory cells were seen when treated with 23% Alcohol based mouthwash.[28] In addition, alcohol containing mouthwashes are reported to have adverse effects on oral structures and functions, these include burning mouth, drying of the oral mucosa, softening effects on composite filling materials and mucosal pain.[58-59]

The concern over the alcohol content of mouthwashes has led to the development of alcohol-free preparations.

In a study of Jose et al, after 6 months of using the alcohol containing mouth rinse no case of nuclear alteration and no DNA damage was seen .[60]

The concentration of alcohol used in the mouthwash lags behind the optimum concentration of 50% to 70% at which alcohol is able to exert its antiseptic effect, hence except for its use as a solvent, alcohol in the mouthwash does not contribute to any other therapeutic effect. Due to this reason, alcohol free mouthwashes in the clinical trials have proven to be as effective as alcohol based mouthwashes, with the former having lesser side effects.[61]

Studies and case reports link a high percentage of alcohol in certain mouthwashes with development of leukoplakia, the lesion was reversible when the mouthwash was stopped.[62]

Present study was conducted to know whether and to what extent commercially available mouth-rinses containing alcohol and alcohol free have direct effect, if any, on the clinical, and cellular level of the oral mucosa. The result was evaluated

clinically and cytological after 2 months use of alcohol (Listerine and Eludril) and alcohol free (Rexidine plus and Hexidine) mouth rinses.

Patients were properly instructed regarding the use of mouth rinse twice daily for 2 months as per the recommendations of the product.

I. CLINICAL PARAMETERS –

The clinical parameters for Plaque index (PI) and Gingival index (GI) were assessed & compared at baseline & after 2 months. The level of plaque index and gingival index for all four mouth rinses AM and AFM reduced significantly but the reduction was found to be insignificant, so AM and AFM were equally effective as an antiplaque and anti-gingivitis agent. The significant reduction in PI and GI of alcohol containing mouth rinse was in accordance with the study done by L. T. Arunachalam, S. Merugu, and U. Sudhakar (2012) in which they have concluded that the addition of alcohol serves many purposes: as a vehicle, as an antiseptic, to stabilize certain active ingredients and also to improve the shelf-life of the product. The accepted concentration of alcohol content in CHX mouthrinses by the FDA is 11.6%. Although Eludril has 42.7% alcohol while Listerine has 21.6% alcohol while Non-alcohol-containing CHX have fewer side-effects, but are less-effective. [63]

So both AM and AFM are equally effective and can be used as an anti-plaque and anti-gingivitis agent.

II. Oral mucosal parameters

Kowitz et al. (1976) described some adverse effects after using mouth washes containing alcohol , such as epithelium desquamation, ulcerations, gingivitis, and

petechiae.[10] but in our study no possible oral mucosal changes were seen after using AM and AFM.

III. CYTOLOGICAL:

Biosafety analysis of two alcoholic (Listerine and Eludril) and non-alcoholic (Rexidine plus and Hexidine) mouth rinses was done using standard endpoints of cytotoxicity viz., Tetrazolium bromide salt MTT assay, Neutral red uptake assay and Trypan blue dye exclusion assay while DNA damage was done usingMicronucleus Assay andChromosomal Aberration (CA) in primary cultures of oral mucosal cells obtained from human volunteers enrolled in the study.

MTT assay:

Results findings of MTT assay was, comparatively, there was more loss of percent cell viability in the cells isolated from the buccal cavity of volunteers using alcohol containing mouth wash than the volunteers using alcohol free mouth rinse , but this loss was not reached to the level of cytotoxicity. Cells allowed to grow for longer period (48-96 h) in culture medium supplemented with growth factors were showing a recovery trend i.e., the loss of percent cell viability got reduced in subsequent incubations of 48, 72 and 96 h and reached near to the basal level. Although the percent cell viability of hexidine during 24 hr shows that the cells are under physiological stress which is called as cytostatic changes, while over a period of time the stress recovered as reflected in the recovery of cell viability. As such there was no significant reduction in percent cell viability reported in the cells following the exposure to all four mouth rinses used in the study.

NRU assay:

The observations of NRU assay were having similar trends as that to the MTT assay . However, the values of percent surviving cells in all the four mouth washes and at all the time points were comparatively higher than that of MTT assay. At 24 h of exposure, few values were more than 100%, which is an indication of physiological stress in the cells due to excessive intracellular activities for maintaining the homeostasis against the xenobiotic exposure. But, the values came down towards the basal levels in the subsequent incubation time points i.e., 48-96 h, an indication of recovery of cellular responses under favorable growth conditions.

Trypan blue dye exclusion assay:

The direct loss in the viable cell count was also assessed at various time points (24, 48, 72 and 96 h) in the cells growing in culture medium following the exposure to four mouth washes used in the study. The values received from the cells isolated from the volunteers during their first visit were considered as 100% to compare the values obtained for live cells from follow-up volunteers after two months of time. In tune with the other assays i.e., MTT and NRU, the loss of viability in cells of Trypan blue dye exclusion assay was insignificant even in first 24 h of culture and loss was further reduced in subsequent incubations i.e., 48-96 h .

Micronucleus Assay: Micronucleus assay was done to assess the accumulation of genetic damage in the cells. The cells were grown in DMEM/F-12 medium alone. There was no increase in the micronucleus (MN) frequency in cells exposed to any of mouth wash. The minor decrease in 96 hr might be due to auto recovery in the cells,and this change during 96 hr are very obvious and routinely observed when we

are using biologically system from human volunteers of different age , gender , ethnic group etc.

Chromosomal Aberration (CA):

The trends were similar as that to micronucleus assay. As such there was no chromosomal aberration observed in any of the group.

Bio-safety analysis of drugs and chemicals in the relevant cells using MTT, NRU, trypan blue dye exclusion assays is a gold standard method and widely accepted as pre-screening in target specific cells host.[64-65] These cells based assays in drug discovery are well accepted as they are suggested as high-throughput screening tools to reduce the time [66-67] In the present investigations, we none of the mouth washes were found to toxic to the cells when isolated and cultured in the culture medium under standard laboratory conditions. The initial decrease in percent cell viability might be due to the physiological stress in the cells outside natural habitat i.e., buccal cavity, which recovered over a period of time i.e., 48-96 h. The other reason of improving in percent cell viability might be the auto-recovery against the stress caused due to the exposure of test materials. The minor decrease in 96 hr might be due to auto recovery in the cells, and this change during 96 hr are very obvious and routinely observed when we are using biologically system from human volunteers of different age , gender , ethnic group etc.

Micronucleus (MN) formation is an indication of fragmentation in chromosomes, and that fragment is not incorporated into daughter nuclei during mitosis, while chromosome aberration (CA) is referred as a missing, extra, or an irregular portion of chromosomal DNA, which is not integrated into daughter nuclei. The xenobiotics exposure had been demonstrated to from an atypical number of chromosomes or a

structural abnormality in one or more chromosomes due to MN and/ or CA. Our findings demonstrated that the all the test materials i.e., alcoholic and non-alcoholic mouth washes have no mutagenic potential even up-to an incubation period of 96 h. However, there was a time dependent induction of MN and CA in cells exposed to positive control i.e., 2mM concentration of ethyl methyl sulphonate. Positive control (2mM concentration of ethyl methyl sulphonate) used in the study has shown significant induction of MN & CA as anticipated since these compound is known to induce genetic damages in the mammalian cells. When we compare our data with positive control the values were statistically and biologically lower then the threshold value. Hence the oral mouth rinses i.e AM and AFM were found to be non – genotoxic under experimental conditions.

Though, the experiments have not been carried out to study the specific binding of the test materials with the receptor or cytosolic receptor of the cells. That could be helpful to elaborate our hypothesis towards the exact mechanism(s) of cell-drug interaction and reasons of no significant toxicity. So, under the experimental conditions, our findings showing no toxic responses in the target cells may reveal the suitability of these mouth washes. The results of present investigations carried out in human buccal mucosal cells identify the test materials biologically safe in human beings.

Conclusion

Based on the clinical observations and cytological assessment, the following conclusions can be drawn in the present study:

1. Plaque index and Gingival index scored at baseline & after 2 months showed statistically significant reduction in both AM and AFM group so they are equally effective.

2. No possible oral mucosal changes i.e epithelial desquamation, ulceration and petechiae were noticed after the use of any of mouth rinses after 2 months.

3. Cytological assessment viz. cytotoxicity assessed by using MTT Assay, NRU Assay and Trypan Blue Assay whereas DNA damage assessed by using Micronucleus Assay and Chromosomal Aberration Assay showed that the changes in the cells after 2 months use of AM and AFM do affect percent cell viability but has not reached to the level of cytotoxicity or genotoxicity.

4. Alcohol containing mouth rinses affected the cell cytology more when compared to non alcoholic mouth rinses but the difference was non significant.

This study concludes that both nonalcoholic as well as alcoholic mouth rinses are equally effective in controlling the plaque. Alcoholic mouth rinses caused more of the cell damage as compared to nonalcoholic mouth rinses but none reached the level of cytotoxicity over two month time period. Both the groups can be used as mouth rinse in non surgical cases. Since they result in some degree of cell damage , it may affect the healing of tissue in surgical cases therefore it should be used judiciously during and after surgical procedures.

Therefore mouth rinses should be prescribed for a certain period of time. Further longitudinal studies with larger sample size and longer duration are required to assess the cytological changes by regular use of mouth rinses.

Bibliography

1. Jackson RJ. Metal salts, essential oils and phenols--old or new?.Periodontol 2000 1997; 15:63-73.

2. Heasman PA, Seymour RA. Pharmacological control of periodontal disease.1. Anti-plaque agents. Journal of Dentistry 1994; 22:323-35.

3. Addy M. Chlorhexidine compared with other locally delivered anti-microbials. A short review. J ClinPeriodontol 1986; 13(10): 957-64.

4. Richards A, Fejerskov O, Larsen J. Fluoride concentrations in dentifrices in relation to efficacy side effects and salivary clearance. Oxford Medical Publications 1992;33: 73-90.

5. Baehni PC, Takeuchi Y. Anti-plaque agents in the prevention of biofilm associated oral diseases. Oral Dis 2003;9:23-29.

6. Council on Dental Therapeutics. J Am Dent Assoc 1986;112:529-32.

7. Bhatti SA, Walsh TF, Douglas CW. Ethanol and ph levels of proprietary mouth rinses. Community Dent Health 1994; 11(2): 71-4.

8. Gьrgan CA, Zaim E, Bakirsoy I, Soykan E. Short-term side effects of 0.2% alcohol-free chlorhexidinemouthrinse used as an adjunct to non-surgical periodontal treatment: a double-blind clinical study. J Periodontol 2006;77:370-84.

9. Madan PD, Sequeira PS, Shenoy K, Shetty J. The effect of three mouthwashes on radiation-induced oral mucositis in patients with head and neck malignancies: a randomized control trial. J Cancer Res Ther. 2008;4(1):3-8.

10. Kowitz GM, Lucatorto FM, Cherrick HM. Effects of mouth-washes on the oral soft tissues. J Oral Med 1976; 31(2): 47-50.

11. Bernstein ML. Oral mucosal white lesions associated with excessive use of Listerine mouthwash. Report of two cases. Oral Surg Oral Med Oral pathol 1978; 46(6):781-5.

12. Bagan JV, Vera-Sempere F, Marzal C, Pellin-Carcelin A, Martin-Bonmatin E, Bagan L. Cytological changes in the oral mucosa after use of a mouth rinse with alcohol: A prospective double blind control study. Med Oral Patol Oral Cir Bucal 2012; 17(6):956-61.

13. Ross- Llor, Lopez-Jornet. Cytogenetic analysis of oral mucosal cells induced by Chlorhexidine, essential oils in ethanolic solution and triclosan mouthwashes. Environ Res 2014; 132:140-5.

14. SmigelK.High alcohol mouthwashes are under scrutiny. J Natl Cancer Inst 1991;*83*: 751-56.

15. R. Shashikala, A. P. Indira,G. S. Manjunath, K. Arathirao, and B. K. Akshatha.Role of micronucleus in oral exfoliative cytology.J Pharm BioalliedSci 2015; 7: 409–13.

16. Reidy JT, McHugh EE, Stassen LF. A review of the role of alcohol in the pathogenesis of oral cancer and the link between alcohol-containing mouthrinses and oral cancer. J Dent Assoc. 2011 ;57:200–2.

17. Rothenstein AS, Picozzi A, Doyle JL, CancroLP, SingerEJ. Soft tissue responses to high frequency use of experimental and commercial mouthwashes. PharmacolTher Dent 1978; 3(1):25-9.

18. Bernstein, Mark L.Oral mucosal white lesions associated with excessive use of Listerine mouthwash.Oral Surgery, Oral Medicine, Oral Pathology 1978; 46 : 781 – 85.

19. J.M Gordon, I.B Lamster, M.C Seiger. Efficacy of Listerine antiseptic in inhibiting the development of plaque and gingivitis.J ClinPeriodontol1985 ;12(8):697-704.

20. DePaola LG, Overholser CD, Meiller TF, Minah GE, Niehaus C.Chemotherapeutic inhibition of supragingival dental plaque and gingivitis development.J ClinPeriodontol 1989;16(5):311-5.

21. Deborah M. Winn, William J. Blot, Joseph K. McLaughlin, Donald F. Austin, Raymond S.Mouthwash Use and Oral Conditions in the Risk of Oral and Pharyngeal Cancer.Cancer Res 1991;51:3044-47.

22. Kayo Kuyama and Hirotsugu Yamamoto.A study of effects of mouthwash on the human oral mucosae: With special references to sites, sex differences and smoking. J Nihon UnivSch Dent.1997;39(4):202-10.

23. Jones CG. Chlorhexidine: is it still the gold standard?Periodontol 2000. 1997;15:55-62.

24. Gьrgan S, Onen A, Kцrtьlь H.In vitro effects of alcohol-containing and alcohol-free mouthrinses on microhardness of some restorative materials.J Oral Rehabil.1997;24(3):244-6.

25. Moghadam BK, Gier R, Thurlow T.Extensive oral mucosal ulcerations caused by misuse of a commercial mouthwash.Cutis. 1999;64(2):131-4.

26. Norppa H, Falck GC.What do human micronuclei contain?Mutagenesis.2003;18(3):221-33.

27. Ciancio S.Improving oral health: current considerations J ClinPeriodontol 2003;30:4-6.

28. PoggiP.Rodriquez y Baena R, Rizzo S, Rota MT. Mouth-rinses with alcohol: cytotoxic effects on human gingival fibroblasts in vitro. J Periodontol 2003; 74(5):623-9.

29. Kristen U, Friedrich RE.Toxicity screening of mouthwashes in the pollen tube growth test: safety assessment of recommended dilutions of twenty brands.In Vivo 2004; 18(6): 803–807.

30. Camila Lopes Cardoso, RenataFalchete do Prado, LuisAntônio de AssisTaveir. Macroscopic and microscopic study of tissue response to oral antiseptics and its influence on carcinogenesis.J. Appl. Oral Sci 2005;13(3): 286-90.

31. Barnett ML.The rationale for the daily use of an antimicrobial mouthrinse.J Am Dent Assoc. 2008;139(3):252.

32. Bernstein ML, Carlish .Mouthrinses containing alcohol and oral cancer. Revision of epidemiological studies. Braz Oral Res 2007;21:16-22.

33. Mala Kamboj, SumitaMahajan.Micronucleus—an upcoming marker of genotoxic damage.Clinical Oral Investigations 2007; 11: 121.

34. Kerr AR, Katz RW, Ship JA.A comparison of the effects of 2 commercially available nonprescription mouthrinses on salivary flow rates and xerostomia.Quintessence Int. 2007;38(8):440-7.

35. Erdemir EO, Sengun A, Ulker M. Cytotoxicity of mouth-rinses on epithelial cells by Micronucleus Test. Eur J Dent 2007; 1(2):80-5.

36. McCullough MJ, Farah CS. The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes.Aust Dent J. 2008;53(4):302-5.

37. Silverman S Jr, Wilder R.Antimicrobial mouthrinse as part of a comprehensive oral care regimen. Safety and compliance factors.J Am Dent Assoc. 2006;137:22-26.

38. Celso Augusto Lemos-Jъnior; GermanoEduardo,MiguelVilloria.Reviewed evidence about the safety of the daily use of alcohol-based mouthrinses.Braz Oral Res 2008;22:24-31

39. Muhammad WasifHaq, MehwishBatool, Syed HammadAhsan, Navid Rashid Qureshi.Alcohol use in mouthwash and possible oral health concerns.J Pak Med Assoc 2009;59:186-9.

40. Werner CW, Seymour RA.Are alcohol containing mouthwashes safe? .Br Dent J 2009;28:207-19.

41. Gunsolley JC. Clinical efficacy of Antimicrobial mouth-rinses. J Dent 2010;38:6.10.

42. Isabel Lanzys David Herrera ,Sagrario Santos.Microbiological effects of an antiseptic mouthrinse in irradiated cancer patients.Med Oral Patol Oral Cir Bucal 2011; 1:1036-42.

43. Koschier F, Kostrubsky V, Toole C, Gallo MA.In vitro effects of ethanol and mouthrinse on permeability in an oral buccal mucosal tissue construct.Food ChemToxicol.2011 ;49(10):2524-9.

44. Reidy JT, McHugh EE, Stassen LF.A review of the role of alcohol in the pathogenesis of oral cancer and the link between alcohol-containing mouthrinses and oral cancer.J Ir Dent Assoc.2011; 57(4):200-2.

45. Tьrkez H, Togar B, Arabaci T.Evaluation of genotoxicity after application of Listerine(R) on human lymphocytes by micronucleus and single cell gel electrophoresis assays.ToxicolInd Health. 2012;28(3):271-5.

46. Carlin V, Matsumoto MA, Saraiva PP, Artioli A, Oshima CT, Ribeiro DA. Cytogenetic damage induced by mouth- rinses formulations in vivo and in vitro. Clin Oral Investiq 2012; 16(3):813-20.

47. Goutham BS, Manchanda K, Sarkar AD, Prakash R, Jha K, Mohammed S. Efficacy of two commercially available Oral Rinses - Chlorhexidine and Listerine on Plaque and Gingivitis - A Comparative Study. Journal of International Oral Health JIOH 2013; 5(4):56-61.

48. AL Zamora-Perez.Increased Number of Micronuclei and Nuclear Anomalies in Buccal Mucosa Cells From People Exposed to Alcohol-Containing Mouthwash.Drug ChemToxicol 2013; 36 (2): 255-260.

49. Madan PD, Sequeira PS, Shenoy K, Shetty J.The effect of three mouthwashes on radiation-induced oral mucositis in patients with head and neck malignancies: a randomized control trial.J Cancer Res Ther. 2008;4(1):3-8.

50. IreneRos-Llor. Author links open the author workspace.PiaLopez-Jornet.Cytogenetic analysis of oral mucosa cells, induced by chlorhexidine, essential oils in ethanolic solution and triclosan mouthwashes.Environmental Research 2014;132: 140-45.

51. R. Shashikala, A. P. Indira, G. S. Manjunath, K. Arathirao, and B. K. Akshatha.Role of micronucleus in oral exfoliative cytology.J Pharm Bioallied Sci. 2015; 7: 409–413.

52. Jose, A. Butler, D. Payne, R. Maclure, P. Rimmer.A randomised clinical study toevaluate the efficacy of alcohol-free or alcohol-containing mouthrinses with chlorhexidine on gingival bleeding.Br Dent J.2015;219(3):125-30.

53. Locci P, Lumare A, Marinucci L.Cytotoxicity of three commercial mouthrinses on extracellular matrix metabolism and human gingival cell behavior.Toxicolln Vitro. 2016;34:88-96.

54. Amini P, Araujo MW, Wu MM, Charles CA, Sharma NC. Comparative antiplaque and antigingivitis efficacy of three antiseptic mouthrinses: a two week randomized clinical trial. Braz Oral Res. 2009;23:319-25.

55. Soames JV. Oral Pathology. In Soames JV: Oral epithelial tumors, melanocytic naevi, and malignant melanoma. 4th ed. Karachi: Oxford University Press 2005; pp 139

56. . Lemos CA Jr, Villoria GE. Reviewed evidence about the safety of the daily use of alcohol-based mouthrinses. Braz Oral Res. 2008;22Suppl 1:24-31.

57. Burket. Oral Medicine. In: Epstein J, Wall IVD. Oral Cancer.11th ed. Ontario: Elsevier 2008; pp 154.

58. Settembrini L, Penugonda B, Scherer W, Strassler H, Hittelman E. Alcohol containing mouthwashes: effects on composite colour. Oper Dent 1995; 20: 14–17.

59. Weiner R, Millstein P, Hoang E, Marshall D. The effect of alcoholic and non-alcoholic mouthwashes on heat-treated composite resins. Oper Dent 1997;22: 249–253.

60. Jose V. Bagan, Francisco Vera-Sempere , Cristina Marzal , Ana Pellın-Carcelйn , EzequielMartнBonmatн, Leticia Bagan. Cytological changes in the oral mucosa after use of a mouth rinse with alcohol: A prospective double blind control study. Med Oral Patol Oral Cir Bucal 2012;17 (6):956-61.

61. Bahna P, Hanna HA, Dvorak T, Vaporciyan A, Chambers M, Raad I. Antiseptic effect of a novel alcohol free mouthwash: a convenient prophylactic alternative for high-risk patients. Oral Oncol 2007; 43:159-64.

62. Weaver A, Fleming SM, Smith DB. Mouthwash and oral cancer: cariconogen or coincidence? J Oral Surg 1979; 37:250-3.

63. A. Jose, A. Butler, D. Payne, R. Maclure. A randomised clinical study to evaluate the efficacy of alcohol-free or alcohol-containing mouthrinses with chlorhexidine on gingival bleeding.Br Dent J.2015 ;219(3):125-3.

64. Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, Silber PM. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. ChemBiol Interact 1999; 121: 17-35.

65. Heussner AH, Dietrich DR, O' Brien E. (2005). In vitro investigation of individual and combined cytotoxic effects of ochratoxin A and other selected mycotoxins on renal cells. Toxicol. In Vitro 2005; 20: 332-341

66. White RE. High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. Annu Rev PharmacolToxicol 2000; 40: 133-157.

67. Ni SS, Mothersill C, Sheehan D, O'Brien NM, O' Halloran J, Van Pelt FN, Davoren M. In vitro cytotoxicity testing of three zinc metal salts using established fish cell lines. ToxicolIn Vitro 2004; 18: 365-376.

Appendices

APPENDIX – I

ETHICAL COMMITTE APPROVAL FORM

Babu Banarasi Das University
Babu Banarasi Das College of Dental Sciences,
BBD City, Faizabad Road, Lucknow – 226028 (INDIA)

Dr. Lakshmi Bala
Professor and Head Biochemistry and
Member-Secretary, Institutional Ethics Committee

Communication of the Decision of the IIIrd Institutional Ethics Sub - Committee

IEC Code: 03

BBDCODS/05/2016

Title of the Project: Alteration in oral mucosal cells using alcohol free and alcohol containing mouth rinses: A Clinico-Cytological Study.

Principal Investigator: Dr. Vandana Gupta

Name and Address of the Institution: BBD College of Dental Sciences Lucknow. **Department:** Periodontology

Type of Submission: New, MDS Project Protocol

Dear Dr. Vandana Gupta,

The Institutional Ethics Sub- Committee meeting comprising following four members was held on 03rd May, 2016.

1.	Dr. Lakshmi Bala Member Secretary	Prof. and Head, Department of Biochemistry, BBDCODS, Lucknow
2.	Dr. Narendra Kumar Gupta Member	Prof., Department of Prosthodontics, BBDCODS, Lucknow
3.	Dr. Smita Govila Member	Reader, Department of Conservative Dentistry, BBDCODS, Lucknow
4.	Dr. Subhash Singh	Reader, Department of Pedodontics, BBDCODS, Lucknow

The committee reviewed and discussed your submitted documents of the current MDS Project Protocol in the meeting.
The proposal was reviewed, comments were communicated to PI thereafter it was revised.

Decisions: The committee approved the above protocol from ethics point of view.

Lakshmi Bala 12/05/16

(Dr. Lakshmi Bala)
Member-Secretary
IEC

Member-Secretary
Institutional Ethic Committee
BBD College of Dental Sciences
BBD University
Faizabad Road, Lucknow-226028

Forwarded by:

(Dr. Vivek Govila)
Principal
BBDCODS

PRINCIPAL
Babu Banarasi Das College of Dental Sciences
(Babu Banarasi Das University)
BBD City, Faizabad Road, Lucknow-226028

APPENDIX – II

CASE SHEET

BABU BANARASI DAS COLLEGE OF DENTAL SCIENCES, LUCKNOW

"ALTERATIONS IN ORAL MUCOSAL CELLS USING ALCOHOL FREE AND ALCOHOL CONTAINING MOUTH-RINSES: A CLINICO-CYTOLOGICAL STUDY"

CLINICAL EVALUATION:

NAME: AGE: SEX:

OPD No.: DATE:

ADDRESS:

Chief complaint:

History of present illness:

Past dental history:

Past medical history:

History of medication:

CLINICAL EVALUATION:

I. Gingiva

Color

Consistency

Size

Position

Bleeding

Suppuration

II. Examination of teeth:

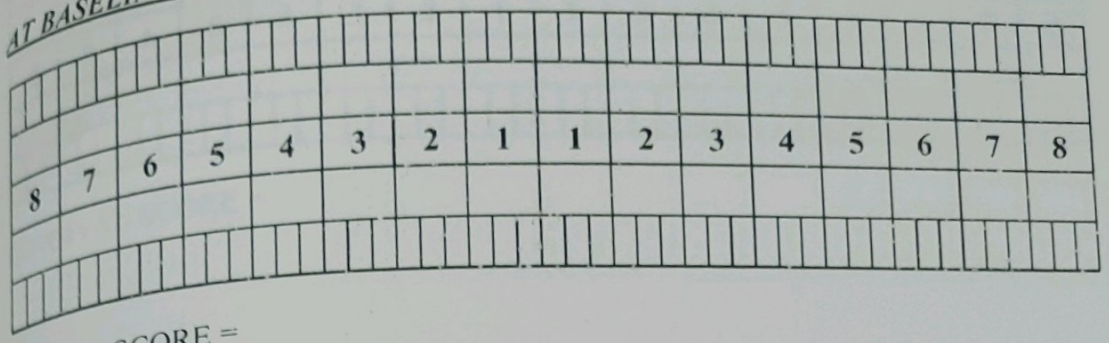
Number of teeth present

Mobility

CLINICAL PERIODONTAL PARAMETERS

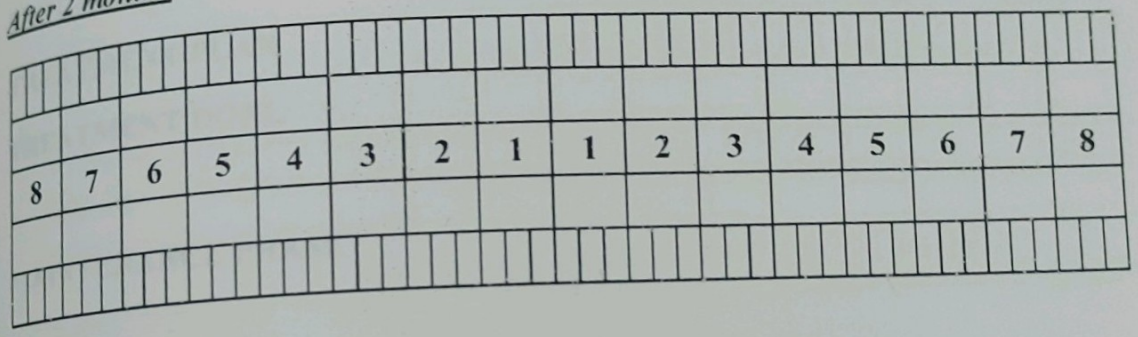
PLAQUE INDEX (Silness & Loe)

AT BASELINE



PLAQUE SCORE =

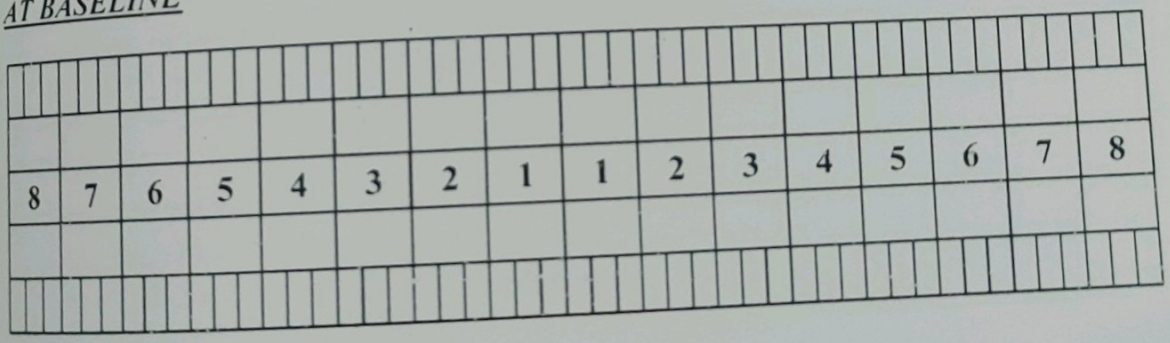
After 2 months



PLAQUE SCORE =

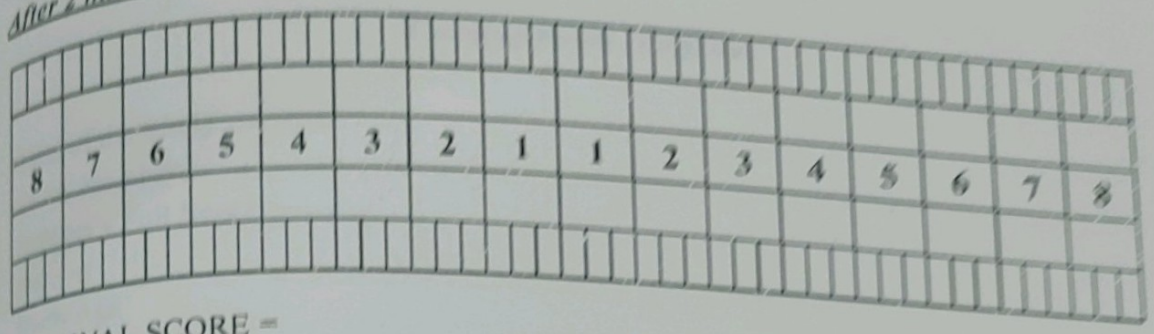
GINGIVAL INDEX (Loe & Silness)

AT BASELINE



GINGIVAL SCORE =

After 2 months



GINGIVAL SCORE =

DIAGNOSIS

PROGNOSIS

TREATMENT PLAN

TREATMENT DONE

MAINTENANCE PHASE

APPENDIX - III

Consent Form (English)

Title of the Study ....
Study Number.....
Subject's Full Name.....
Date of
Birth/Age
.....
Address.....
.....

Phone no. and e-mail address.....

1. I confirm that I have read and understood the Participant Information Document dated .....for the above study and have had the opportunity to ask questions. **OR** I have been explained the nature of the study by the Investigator and had the opportunity to ask questions.
2. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.
3. I understand that the sponsor of the project, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. However, I understand that my identity will not be revealed in any information released to third parties or published.
4. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).
5. I permit the use of stored sample (tooth/tissue/blood) for future research.
   Yes [ ] No [ ] Not Applicable [ ]
6. I agree to participate in the above study. I have been explained about the complications and side effects, if any, and have fully understood them. I have also read and understood the participant/volunteer's Information document given to me.

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative.....

Signatory's Name..... Date .....

Signature of the Investigator..... Date.....

Study Investigator's Name..... Date.....

Signature of the witness..... Date.....

Name of the witness.....

Received a signed copy of the PID and consent form

Signature/thumb impression of the subject or legally Acceptable representative Date.....

APPENDIX - IV

Participant Information Document (PID)

1. Study Title

Alterations In Oral Mucosal Cells Using Alcohol Free And Alcohol Containing Mouth-Rinses: A Clinico-Cytological Study

2. Invitation Paragraph

You are being invited to take part in a research study, it therefore is important for you to understand why the study is being done and what it will involve. Please take time to read the following information carefully. Ask us for any clarifications or further information. Whether or not you wish to take part is your decision.

3. What is the purpose of the study?

The purpose of study is to find out whether and to what extent alcohol mouth rinse and alcohol free mouth rinses are safe. .

4. Why have I been chosen?

You have been chosen for this study as you are fulfilling the required criteria for the diseased condition.

5. Do I have to take part?

Your participation in the research is entirely voluntary. If you do, you will be given this information sheet to keep and will be asked to sign a consent form. During the study you still are free to withdraw at any time and without giving a reason.

6. What will happen to me if I take part?

This study will last for 2 months and you will be recalled after 2 months.

7. What do I have to do?

You do not have to change your regular lifestyles for the investigation of the study. This research study is self-sponsored by the candidate. You do not have to pay for any procedures involved.

8. What is the procedure that is being tested?

Alterations in oral mucosal cell by use of alcohol containing and alcohol free mouth rinses.

9. What are the interventions for the study?

The study includes treatment with different mouthwashes, along with scaling and root planing.

10. What are the side effects of taking part?

There are no side effects on patients of this study.

11. What are the possible disadvantages and risks of taking part?

There are no possible disadvantages for the patients of this study.

12. What are the possible benefits of taking part?

Your diseased condition will be eliminated efficiently.

13. What if new information becomes available?

If additional information becomes available during the course of the research you will be told about these and you are free to discuss it with your researcher, your researcher will tell you weather you want to continue in the study. If you decide to withdraw, your researcher will make arrangements for your withdrawal. If you decide to continue in the study, you may be asked to sign an updated consent form.

14. What happens when the research study stops?

If the study stops/finishes before the stipulated time, this will be explained to the patient/volunteer.

15. What if something goes wrong?

If any severe adverse event occurs, or something goes wrong during the study, the complaints will be handled by reporting to the institution (s), and IEC.

16. Will my taking part in this study be kept confidential?

Yes it will be kept confidential.

17. What will happen to the results of the research study?

The results of the study may be used to provide data of the periodontal health status and the treatment needs in this region of India for planning of further large scale studies. Your identity will be kept confidential in case of any report/publications.

18. Who is organizing the research?

This research study is self-sponsored by the candidate. You do not have to pay for any procedures involved.

19. Will the results of the study be made available after study is over?

Yes.

20. Who has reviewed the study?

The study has been reviewed and approved by the Head of the Department and the IEC of the institution.

21. Contact for further information

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APPENDIX-IV

TABLES OF CLINICAL & CYTOLOGICAL PARAMETERS

LISTERINE

	PI		GI	
SNO	Baseline	2 month	Baseline	2 month
1	1	0	2	1
2	1	0	2	0
3	1	1	2	0
4	1	0	1	0
5	1	0	1	0
6	0	1	1	0
7	0	0	1	0
8	1	0	2	0
9	1	0	1	0
10	0	1	1	0
11	1	0	2	1
12	1	1	1	0
13	2	0	1	0
14	1	1	2	1
15	0	0	1	0
16	0	0	1	1
17	1	0	1	0
18	1	1	1	0
19	0	0	2	0
20	1	0	2	1
21	1	1	2	0
22	2	0	2	0
23	0	1	2	1
24	1	1	2	0
25	0	0	1	1
26	0	0	1	1
27	1	0	1	1
28	1	1	1	0
29	0	0	1	1
30			0	0

ELUDRIL

S.NO.	GI		PI	
	Baseline	2 month	Baseline	2 month
1	1	0	2	1
2	1	1	2	0
3	1	0	1	0
4	1	0	1	0
5	1	1	1	0
6	0	0	1	0
7	0	0	2	2
8	1	0	1	0
9	1	1	1	0
10	0	0	2	1
11	1	0	1	0
12	1	1	1	1
13	2	0	2	1
14	1	1	1	1
15	0	0	1	1
16	0	0	1	0
17	1	0	1	0
18	1	1	2	0
19	0	0	2	1
20	1	0	2	0
21	1	1	2	2
22	2	0	2	1
23			2	0
24	1	1	1	1
25	0	0	1	1
26	0	0	1	1
27	1	0	1	1
28	1	1	1	1
29	0	0	0	0
30				

FLUDRIL

S.NO.	GI		PI	
	Baseline	2 month	Baseline	2 month
1	1	0	2	1
2	1	1	2	0
3	1	0	2	0
4	1	0	1	0
5	1	1	1	0
6	0	0	1	0
7	0	0	1	0
8	1	0	2	2
9	1	1	1	0
10	0	0	1	0
11	1	0	2	1
12	1	1	1	0
13	2	0	1	1
14	1	1	2	1
15	0	0	1	1
16	0	0	1	1
17	1	0	1	0
18	1	1	1	0
19	0	0	2	0
20	1	0	2	1
21	1	1	2	0
22	2	0	2	2
23			2	1
24	1	1	2	0
25	0	0	1	1
26	0	0	1	1
27	1	0	1	1
28	1	1	1	1
29	0	0	1	1
30			0	0

REXIDINE PLUS

S.NO.	GI		PI	
	Baseline	2 month	Baseline	2 month
1	1	0	1	1
2	1	0	2	2
3	1	0	2	1
4	1	0	2	1
5	1	0	2	1
6	1	0	1	1
7	1	1	1	1
8	1	0	0	0
9	1	1	0	0
10	1	0	0	0
11	1	1	2	1
12	1	0	1	1
13	1	0	1	0
14	1	0	1	0
15	2	0	1	1
16	2	0	0	0
17	1	1	0	0
18	0	0	0	0
19	0	0	2	1
20	0	0	1	0
21	1	0	1	1
22	0	0	1	1
23	0	0	1	1
24	0	0	2	0
25	1	0	1	1
26	1	1	1	1
27	1	0	0	0
28	2	0	1	1
29	1	0	2	1
30			1	0

S.NO.	GI		PI	
	Baseline	2 month	Baseline	2 month
1	1	0	1	0
2	1	0	2	2
3	1	0	2	1
4	1	0	2	1
5	1	0	1	0
6	1	0	1	1
7	1	1	0	1
8	1	0	0	0
9	1	1	0	0
10	1	1	2	1
11	1	1	1	1
12	1	0	1	0
13	1	0	1	0
14	1	0	1	1
15	2	0	0	0
16	2	0	0	0
17	1	1	0	0
18	0	0	2	1
19	0	0	1	0
20	0	0	1	1
21	1	1	1	1
22	0	0	1	1
23	0	0	2	0
24	0	0	1	1
25	1	0	1	1
26	1	1	0	0
27	1	0	1	1
28	2	0	2	0
29	1	0	1	0
30				

MTT Assay

				AVG	SD	SE	P value
24 h							
LISTERINE	77	80	74	77	3	1.73	P < 0.01
ELUDRIL	79	86	74	79.6667	6.02771	3.48	P > 0.05
REXIDINE	91	96	86	91	5	2.89	P > 0.05
HEXIDINE	103	105	101	103	2	1.15	P > 0.05
MgCl2 (10	79	82	76	79	3	1.73	P < 0.01

				AVG	SD	SE	P value
48 h							
LISTERINE	82	89	91	87.3333	4.72582	2.73	P < 0.01
ELUDRIL	85	89	81	85	4	2.31	P < 0.01
REXIDINE	91	94	88	91	3	1.73	P < 0.01
HEXIDINE	99	101	97	99	2	1.15	P > 0.05
MgCl2 (10	67	70	64	67	3	1.73	P < 0.01

				AVG	SD	SE	P value
72 h							
LISTERINE	90	92	95	92.3333	2.51661	1.45	P < 0.01
ELUDRIL	92	95	89	92	3	1.73	P < 0.01
REXIDINE	98	99	91	96	4.3589	2.52	P < 0.01
HEXIDINE	99	97	99	98.3333	1.1547	0.67	P < 0.01
MgCl2 (10	43	47	39	43	4	2.31	P < 0.01

				AVG	SD	SE	P value
96 h							
LISTERINE	95	97	97	96.3333	1.1547	0.67	P < 0.01
ELUDRIL	96	95	99	96.6667	2.08167	1.2	P < 0.01
REXIDINE	99	99	96	98	1.73205	1	P < 0.01
HEXIDINE	99	97	99	98.3333	1.1547	0.67	P < 0.01
MgCl2 (10	30	33	27	30	3	1.73	P < 0.01

				AVG	SD	SE	P value
	81	85	77	81	4	2.31	P < 0.01
	87	82	91	86.6667	4.50925	2.6	P > 0.05
	102	107	97	102	5	2.89	P > 0.05
	105	108	102	105	3	1.73	P > 0.05
	81	84	78	81	3	1.73	P < 0.01

				AVG	SD	SE	P value
	90	87	85	87.3333	2.51661	1.45	P < 0.01
	91	95	87	91	4	2.31	P < 0.05
	99	102	96	99	3	1.73	P > 0.05
	101	105	97	101	4	2.31	P > 0.05
	69	71	67	69	2	1.15	P < 0.01

				AVG	SD	SE	P value
	92	96	90	92.6667	3.05505	1.76	P < 0.01
	93	95	97	95	2	1.15	P < 0.01
	98	98	96	97.3333	1.1547	0.67	P < 0.01
	99	97	97	97.6667	1.1547	0.67	P > 0.05
	47	50	44	47	3	1.73	P < 0.01

				AVG	SD	SE	P value
	99	99	103	100.333	2.3094	1.33	P < 0.01
	95	99	97	97	2	1.15	P < 0.01
	99	99	95	97.6667	2.3094	1.33	P < 0.01
	99	96	98	97.6667	1.52753	0.88	P < 0.01
	30	33	27	30	3	1.73	P < 0.01

			AVG	SD	SE	%age	
37600	38200	37000	37600	600	346.41	94	P value
37100	38000	36200	37100	900	519.62	92.75	P > 0.05
38800	40100	37500	38800	1300	750.56	97	P > 0.05
38400	39500	37300	38400	1100	635.09	96	P > 0.05
29800	30800	28800	29800	1000	577.35	74.5	P < 0.01

			AVG	SD	SE		
38000	38900	38090	38330	495.681	286.18	95	
37900	38700	37600	38066.7	568.624	328.3	94.75	P > 0.05
38600	40700	41600	40300	1539.48	888.82	96.5	P > 0.05
39200	39900	39000	39366.7	472.582	272.85	98	P > 0.05
25300	26000	24600	25300	700	404.15	63.25	P < 0.01

			AVG	SD	SE		
38700	39300	38670	38890	355.387	205.18	96.75	P value
38100	38800	37900	38266.7	472.582	272.85	95.25	P > 0.05
38700	40900	42100	40566.7	1724.34	995.55	96.75	P > 0.05
40100	40600	40700	40466.7	321.455	185.59	100.25	P > 0.05
19100	20000	18200	19100	900	519.62	47.75	P < 0.01

			AVG	SD	SE		
39300	39900	39200	39466.7	378.594	218.58	98.25	P value
38690	39400	38500	38863.3	474.377	273.88	96.725	P > 0.05
39200	41000	42700	40966.7	1750.24	1010.5	98	P > 0.05
40100	40600	40700	40466.7	321.455	185.59	100.25	P < 0.05
14300	15100	13500	14300	800	461.88	35.75	P < 0.01

Micronucleus Assay

				AVG	SD	SE	
24 h							
LISTERINE	3	4	2	3	1	0.58	P > 0.05
ELUDRIL	5	6	2	4.333333	2.081666	1.2	P > 0.05
REXIDINE PLUS	2	5	3	3.333333	1.527525	0.88	P > 0.05
HEXIDINE	4	2	2	2.666667	1.154701	0.67	P > 0.05
2 mM EMS	17	20	14	17	3	1.73	P < 0.01
48 h				AVG	SD	SE	
LISTERINE	5	4	5	4.666667	0.57735	0.33	P > 0.05
ELUDRIL	4	4	5	4.333333	0.57735	0.33	P > 0.05
REXIDINE PLUS	5	3	2	3.333333	1.527525	0.88	P > 0.05
HEXIDINE	4	3	3	3.333333	0.57735	0.33	P > 0.05
2 mM EMS	23	26	20	23	3	1.73	P < 0.01
72 h				AVG	SD	SE	
LISTERINE	3	7	5	5	2	1.15	P > 0.05
ELUDRIL	4	6	3	4.333333	1.527525	0.88	P > 0.05
REXIDINE PLUS	4	3	2	3	1	0.58	P < 0.05
HEXIDINE	5	2	2	3	1.732051	1	P < 0.01
2 mM EMS	27	31	23	27	4	2.31	P < 0.01
96 h				AVG	SD	SE	
LISTERINE	3	2	5	3.333333	1.527525	0.88	P > 0.05
ELUDRIL	4	2	4	3.333333	1.154701	0.67	P < 0.05
REXIDINE PLUS	4	4	7	5	1.732051	1	P < 0.01
HEXIDINE	4	3	6	4.333333	1.527525	0.88	P < 0.01
2 mM EMS	37	41	33	37	4	2.31	P < 0.01

Chromosomal Aberration Assay

				AVG	SD	SE	
24 h							
LISTERINE	1	1	2	1.333333	0.57735	0.33	P > 0.05
ELUDRIL	2	1	1	1.333333	0.57735	0.33	P > 0.05
REXIDINE PLUS	1	1	2	1.333333	0.57735	0.33	P > 0.05
HEXIDINE	2	2	1	1.666667	0.57735	0.33	P < 0.05
2 mM EMS	17	19	15	17	2	1.15	P < 0.01
48 h				AVG	SD	SE	
LISTERINE	2	2	1	1.666667	0.57735	0.33	P > 0.05
ELUDRIL	1	1	2	1.333333	0.57735	0.33	P > 0.05
REXIDINE PLUS	2	1	1	1.333333	0.57735	0.33	P > 0.05
HEXIDINE	1	1	3	1.666667	1.154701	0.67	P < 0.05
2 mM EMS	21	24	18	21	3	1.73	P < 0.01
72 h				AVG	SD	SE	
LISTERINE	1	2	2	1.666667	0.57735	0.33	P > 0.05
ELUDRIL	2	2	1	1.666667	0.57735	0.33	P > 0.05
REXIDINE PLUS	2	3	3	2.666667	0.57735	0.33	P < 0.05
HEXIDINE	1	2	2	1.666667	0.57735	0.33	P < 0.01
2 mM EMS	31	34	28	31	3	1.73	P < 0.01
96 h				AVG	SD	SE	
LISTERINE	2	2	4	2.666667	1.154701	0.67	P > 0.05
ELUDRIL	2	1	2	1.666667	0.57735	0.33	P > 0.05
REXIDINE PLUS	3	1	1	1.666667	1.154701	0.67	P < 0.05
HEXIDINE	2	1	1	1.333333	0.57735	0.33	P < 0.01
2 mM EMS	39	42	36	39	3	1.73	P < 0.01

APPENDIX-V

FORMULA USED FOR THE STATISTICAL ANALYSIS

The Arithmetic Mean

The most widely used measure of central tendency is arithmetic mean, usually referred to simply as the mean, calculated as

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

The Standard Deviation

The standard deviation (SD) is the positive square root of the variance, and calculated as

$$SD = \sqrt{\frac{\sum X_i^2 - \frac{(\sum X_i)^2}{n}}{n-1}}$$

where, n= no. of observations

Student's t-test

t Test is often called **Student's T test** in the name of its founder "Student". T test is used to compare two different set of values. It is generally performed on a small set of

data. T test is generally applied to normal distribution which has a small set of values. This test compares the mean of two samples. T test uses means and standard deviations of two samples to make a comparison. The formula for T test is given below:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where,

[Math Processing Error] = Mean of first set of values

[Math Processing Error] = Mean of second set of values

S_1 = Standard deviation of first set of values

S_2 = Standard deviation of second set of values

n_1 = Total number of values in first set

n_2 = Total number of values in second set.

$$SE = \sqrt{S^2 X \left[\frac{1}{n_1} + \frac{1}{n_2}\right]}$$

Student's t-test was used to calculate the differences between the means of two groups

where,

$$t = \frac{\bar{X}_1 - \bar{X}_2}{SE}$$

S^2 is the pooled variance and n_1 and n_2 are number of observations in group 1 and 2 respectively. The degrees of freedom (DF) is calculated as

$$DF = n1 + n2 - 2$$

Wilcoxon rank sum test:

Let N be the sample size, the number of pairs. Thus, there are a total of $2N$ data points. For $i = 1,\ldots, N$, let $x_{1,i}$ and $x_{2,i}$ denote the measurements.

H_0: median difference between the pairs is zero

H_1: median difference is not zero.

1. For $i = 1,\ldots, N,$, calculate $|x_{2,i} - x_{1,i}|$ and $\text{sgn}(x_{2,i} - x_{1,i})$, where sgn is the sign function.
2. Exclude pairs with $|x_{2,i} - x_{1,i}| = 0$. Let N_r be the reduced sample size.
3. Order the remaining N_r pairs from smallest absolute difference to largest absolute difference, $|x_{2,i} - x_{1,i}|$.
4. Rank the pairs, starting with the smallest as 1. Ties receive a rank equal to the average of the ranks they span. Let R_i denote the rank.
5. Calculate the test statistic W

$$W = \left| \sum_{i=1}^{N_r} [\text{sgn}(x_{2,i} - x_{1,i}) . R_i] \right|$$

The absolute value of the sum of the signed ranks.

6. As N_r increases, the sampling distribution of W converges to a normal distribution. Thus,

For $N_r \geq 10$, a z-score can be calculated as

$$z = \frac{W - 0.5}{\sigma_w}, \quad \sigma_w = \sqrt{\frac{N_r(N_r + 1)(2N_r + 1)}{6}}$$

If $z > z_{critical}$ then reject H_0

For $N_r < 10$, W is compared to a critical value from a reference table.

If $W \geq W_{critical}$ then reject H_0.

Alternatively, a *p*-value can be calculated from enumeration of all possible combinations of W given N_r.

Level of significance: "p" is level of significance

$p > 0.05$ = Not significant

$p < 0.05$ = Significant

$p < 0.01$ = Highly significant

$p < 0.0001$ = Very highly significant