Chapter 5
Discussion
DISCUSSION

Chlorhexidine is the most commonly prescribed chemical plaque control agent used in the clinical dental practice. It is used as an adjunct to mechanical plaque control measures (Jones, 1997). Dental plaque inhibition by Chlorhexidine was first shown by Schroeder in 1969 but it was Loe & Schiott in the year 1970 who in their study showed that rinsing with 10 ml of 0.2% Chlorhexidine solution inhibited plaque formation and development of gingivitis in humans.

Chlorhexidine is the gold standard of all chemical plaque control measures used in clinical practice and its efficacy as has been proved in several studies (Loe & Schiott, 1970; Gumming & Loe, 1973; Lang & Raber, 1981; Lang et al., 1982; Segreto et al., 1986; Breex et al., 1989; Jones, 1997). Chlorhexidine has bacteriostatic, bactericidal, fungicidal, fungistatic and antiviral properties and therefore has broad spectrum of activity against oral pathogens (Davies et al., 1954). The major advantage of chlorhexidine is its substantivity which means that it remains active in the oral cavity for several hours after its application (Hjeljord et al., 1973).

It has been used in various oral formulations such as mouthrinses, gels, sprays, local drug delivery and as irrigant in full mouth disinfection (Addy & Renton-Harper, 1996; Stanley et al., 1989; Quirynen et al., 2000). These different Chlorhexidine based products have been tested in various studies in last two decade and have supported the basis of its several indications in clinical practice (Addy & Renton-Harper, 1996; Addy & Moran 1997).

There are various side-effects associated with chlorhexidine use such as tooth staining and discoloration, taste alterations, oral ulcerations, parotid swellings and viral infections (Flotra et al., 1971).

Chlorhexidine molecule is a symmetrical molecule having two 4-chlorophenyl ring connected by a central metahexythelene bridge. It is strong base and cationic at physiological pH so has very strong affinity towards oral bacteria which are negatively charged, leading to alteration in permeability of their cell membrane through leakage of potassium and phosphate ions causing cell lysis and death of the bacteria (Davies, 1954; Jones, 1997).
Also, the negatively charged oral epithelial cells come in contact with cationic chlorhexidine molecule which causes cell lysis, DNA damage and cell death at various range of concentrations. CHX induces time dependent toxicity on the cells and also negatively effects DNA synthesis. CHX is cytotoxic to human dermal fibroblasts in-vitro at concentrations 5-2400 times below those used in clinical practice. CHX also causes mitochondrial injury of the oral epithelial cells (Hidalgo & Dominguez, 2001).

CHX binds to eukaryotic plasma membrane of oral epithelial cells by nonspecific binding to negative moieties such as protein and phospholipids and alter the structure of cell membrane and osmotic equilibrium of the cell (Hidalgo & Dominguez, 2001; Babich et al., 1995).

CHX causes alteration in actin cytoskeletal assembly, stimulate apoptosis and autophagic or necrotic cell death, alter mitochondrial membrane potential, induce depletion of intracellular ATP and affect succinate dehydrogenase activity in dermal fibroblasts and trigger intracellular Ca2+ increase leading to ROS generation, thereby playing a critical role for these mediators in the signal transduction cascades underlying the toxicity of CHX in the cells (Hidalgo & Dominguez, 2001; Giannelli et al., 2008).

Several invitro and animal studies have been done to assess the genotoxic and cytotoxic effect of chlorhexidine. One year after establishment of chlorhexidine as antiplaque and antigingivitis agent Hegeland et al., (1971) in their invitro study on human RBC and epithelial cells had first shown CHX toxic effects at very low concentration of 0.05 mM and also observed inhibition of Na K ATPase on the membrane of erythrocytes.

Two years later, Kenney et al.,(1972) showed that 0.2% and 2% Chlorhexidine solutions caused disruption of PMN cell membranes with fixation of the cytoplasmic contents.

Mobacken et al., (1974) in animal study on rats observed decrease in tensile strength of wounds treated with 0.1% and 0.02% Chlorhexidine.

Goldschmidt et al.,(1977) in their in vitro study observed that on exposure of cell cultures of human gingival fibroblasts and HeLa cells to concentration of CHX equal
to or greater than 0.01% (0.2mM) resulted in death of the cells which was measured by release of membrane bound 51 Cr from these cells.

Further, Bassetti & Kallenberger (1980) in their animal study on rats studied the effect of CHX rinsing on wound healing of open mucosal osseous wounds and observed that on increasing the CHX concentrations from 0.1% to 0.2% and 0.5% retarded the wound healing.

Likewise, Gabler et al., (1987) showed in their invitro study found that 0.01% CHX caused very little damage to RBCs and PMNs but on increasing the CHX concentration to 0.02% caused 80-90% lysis of RBCs and staining of PMNs.

Also, Seymour et al., (1990) and Watts et al., (1989) both investigated the effect of Chlorhexidine on PMN chemotaxis and found a dose-related decrease in chemotaxis of the PMN on increasing concentrations from 0.002% to 0.2% in aqueous solutions. Cell lysis and total immobility of PMNs was seen at 0.2% CHX concentrations.

Research has shown that CHX inhibits protein in dose dependent manner. CHX has significant cytotoxic effect on cells in vitro, although various cell functions such as proliferation, collagen gel contraction and protein synthesis were affected to different degrees (Goldschmidt et al., 1977; Pucher & Daniel, 1992).

Cline and Layman (1992) in their invitro study on effect of CHX exposure on growth and attachment of gingival fibroblasts and periodontal ligament to root surface showed that 0.12% CHX did not inhibit attachment of cultured periodontal cells to pretreated roots but direct exposure of much lower concentration of 0.0025% to 0.01% CHX caused dose dependent inhibition of growth.

Similarly, Alleyn et al., (1991) in their invitro study on human gingival fibroblasts exposed to 0.12% CHX demonstrated significant inhibition of fibroblasts attachment leading to deleterious effects in periodontal regeneration and it was also seen that CHX induced a dose-dependent reduction on lymphocyte proliferation (Cline et al., 1992; Mariotti & Rumpf, 1999; Pucher & Daniel, 1992; Arabaci et al., 2013)
Mariotti and Rumpf (1999) in their invitro study showed that CHX exposure for short periods of time (1, 5 and 15 minutes), induce a dose-dependent reduction in cellular proliferation of gingival fibroblasts.

Chen et al.,(2003) demonstrated a 50% reduction in cellular viability of periodontal ligament cells under CHX incubation at a very low concentration (15 mg/ml).

Emmadi et al.,(2008) in their invitro study examined the effect of commercially available mouthrinses on fibroblast proliferation and found that chlorhexidine, Listerine and Povidine iodine induced dose-dependent reduction in cellular proliferation of fibroblasts and CHX showed the highest toxicity at all intervals of exposure.

Further, Bonacorsi et al., (2004) showed that macrophages were sensitive to the toxic effects of chlorhexidine and that the cytotoxic effect of CHX was dependent on the concentration and time of exposure.

Lessa et al. (1992) showed that CHX was cytotoxic to odontoblasts at very low concentrations (0.06–2%).Giannelli et al.,(2008) examined cell viability and cell death in osteoblastic, endothelial and fibroblastic cell lines exposed to various concentrations of CHX (0.0025%, 0.005%, 0.0075%, 0.01% and 0.12%) and showed a significant reduction of cell viability as the concentration of CHX increased.

Almazin et al.,(2009) invitro studied the effect of 10% doxycycline gel, 2.5 CHX Periochip and 1 mg minocycline hydrochloride minocycline hydrochloride microsphere on proliferation and differentiation of osteoblasts employing trypan blue test and found CHX was cytotoxic towards osteoblasts at 0.5mg/ml concentration.

Further, it was seen by Newton et al., (2004) that chlorhexidine releases iron from ferritin. The iron mobilization from ferritin induces oxidative stress leading to lipid peroxydation, and oxidative damage thus chlorhexidine promotes iron induced carcinogenesis (Rocha et al., 2000). Also, Parachloroaniline; degradation product of Chlorhexidine has mutagenic effects(Vander Bijli,1984).

The above evidence gathered about cytotoxicity and genotoxicity of Chlorhexidine has been done in invitro and animals models and therefore to translate the above findings into humans and to shed additional light on the genotoxic effects of
Chlorhexidine on humans we planned our study with the aim of assessing the genotoxic effect of Chlorhexidine mouthrinse on exfoliated buccal epithelial cells in chronic gingivitis patients by Micronucleus test.

Micronucleus test uses micronuclei as a biomarker for measuring the genotoxicity of any chemical or agent and there is great enthusiasm regarding micronuclei as a genetic biomonitooring biomarker (Stich et al., 1992).

Biomarkers have been used in medicine, toxicology and biomonitooring studies to assist in diagnosing, staging and evaluating the risk assessment. There are various biomarker by which we can study the genotoxic effects of various chemicals.

The biomarkers have been classified on three purposes; the first purpose is to give account about deleterious chemical exposure. Secondly, to show the biological effects on target tissue and thirdly to give information about individual susceptibility (Indulski & Lutz, 1997).

There are various assays that have been developed as potential biomarkers in biomonitoring studies which include assessing metaphase chromosomal aberrations, sister chromatid exchanges and host cell reactivation. These methods are quite laborious and time consuming and require highly trained technicians to read and interpret the slides (Stich et al., 1992).

Micronucleus arise from accentric fragments or whole chromosomes which are not included in the main nuclei of the daughter cells. The micronuclei are formed when the cells are exposed to the substances which cause chromosomal breakage (Clastogens) and also affect the spindle apparatus (Aneugens). Micronucleus test in exfoliated buccal mucosa cells have been systematically used in genetic biomonitoring of populations exposed to various genotoxic agents like; tobacco products, pesticides and alcohol consumption (Nersesyan, 2006; Jyoti et al., 2015).

The advantage of Micronucleus is its simplicity, relative ease of scoring, economical in terms of cost and required person-time resources. Moreover, precision is obtained from scoring large number of cells. Micronucleus cell index reflect genomic instability (Neri et al., 2003) and increased frequency of micronuclei indicates risk of malignancy (Hejl et al., 2000). Micro-nucleus is multiple-endpoint test which indicates chromosomal aberration (Kirsch-Volders et al., 1997; Miller et al., 1998).
We have used exfoliated buccal epithelial cells to study the genotoxic effect of chlorhexidine mouthwash as the buccal mucosa is an easily accessible tissue for sampling cells in a minimally invasive manner and does not cause undue stress to the subjects. It is used in molecular epidemiological studies investigating the impact of nutrition, lifestyle factors, genotoxin exposure and genotype on DNA damage and cell death. It provides barrier to potential carcinogen which is metabolized generating potential reactive products. The buccal mucosal cells are of ectodermal origin and 90% of all cancers are of ectodermal origin in humans. Therefore buccal mucosa has been used to monitor early genotoxic events which enter the body either through ingestion or inhalation (Thomas et al., 2009).

The primary objective of our study was to assess genotoxic effect of 0.2% chlorhexidine mouthwash on exfoliated buccal epithelial cells. The secondary objectives of the study were to assess total micronucleated cells and number of micronuclei in exfoliated buccal epithelial cells of chronic gingivitis patient exclusively on mechanical plaque control and with adjunct 0.2% chlorhexidine mouthwash and also to study the effect of duration of 0.2% CHX mouthwash usage on total micronucleated cells and number of micronuclei in buccal epithelial cells as marker of genotoxicity.

In our study we enrolled the patients into; Group A or Control group (n=101) comprised of chronic gingivitis patients who were exclusively on mechanical plaque control measure, where as other Group B or Study group (n=255) comprised of Chronic gingivitis patients who were on mechanical plaque control therapy along with adjunct 0.2% Chlorhexidine gluconate mouthwash twice daily.

The Group B (study group) was further divided into 5 subgroups having 51 subjects each on increasing duration of Chlorhexidine use from ≤1 week to ≤24 weeks into B1, B2, B3, B4 and B5. The buccal cells were collected using a soft toothbrush and were swirled into test tube containing tris buffer and were centrifuged. After centrifugation the buccal cells were collected on glass slide and further treated with ethyl alcohol and stained with 5% Giemsa for staining of micronuclei and 2000 cells were observed per slide(subject) for scoring the number Micronucleated cells and number of Micronuclei.
The age of the subjects ranged from 13 to 73 years in Group A or Control with mean age of 27.89±10.89 where as in the Group B or Study the age ranged from 13 to 70 years with mean age of 31.10±12.06 years. However, the majority of subjects in both the control and study group fell in 21to 40 years age group followed by ≤ 20 years age group [Table 2, Figure 13]. There was no statistically significant difference in age distribution (p=0.443) in control and study groups.

On comparing the mean age among control (Group A) and study subgroups, the mean age of study subgroups B1,B2,B3,B4,B5 were 28.89±10.91, 30.53±10.97, 35.22±13.68, 32.82±13.03, 28.35±10.39 respectively. There was no statistically significant difference (p=0.416) in age distribution among the control and study subgroups. The majority of subjects in the both control and study subgroups belonged to 21-40 years group followed by ≤ 20 years age group[Table 6, Figure 17].

However, in similar study by Erdemir et al.(2007) the age group ranged from 16-24 years whereas in study by Carlin et al.,(2012) the mean age of subjects recruited in the study was 37.3±11.1 years.

On comparing the sex distribution there were 55 males and 46 females in the control (Group A) where as there were 129 males and 126 females in the study group (Group B). The majority of subjects in both control and study groups were males, however there was no significant difference (p=0.510) between both groups and study subgroups in terms of sex distribution [Table 3, Figure 14]. Also, on comparing sex distribution among study subgroups; B1 comprised of 24 males & 27 females; B2 comprised of 27 males & 24 females; B3 comprised of 27 males & 24 females; B4 comprised of 25 males & 26 females; B5 comprised of 26 males & 25 females. The difference of sex distribution among control and study subgroups was not statistically significant (p=0.965). However in a similar study by Carlin et al.,(2012) comprising of 75 subjects there were 45 males and 30 females. In another study by Erdemir et al.,(2007) comprising of 28 subjects had 13 males and 15 females without any significant difference in sex distribution.

On comparing the periodontal health indices, the mean plaque index of patients in Group A( control group) was 2.03±0.49 as compared to 0.60±0.44 for those in the Group B( study group)[Table 4, Figure 15].There was significant difference between
plaque index of control and study group (p<0.001), thus our finding show strong antiplaque action of chlorhexidine mouthwash.

The mean plaque index of patients in study subgroups B1,B2,B3,B4 & B5 were 0.86±0.51, 0.71±0.53, 0.16±0.21, 0.52±0.32, 0.74±0.16 respectively with significant subgroup difference (p<0.001) with subgroup B3 having the lowest mean plaque index.

Likewise, Erdemir et al.,(2007) observed plaque index of 1.41±0.30 before 0.2% chlorhexidine mouthrinse 10 ml twice daily use and was reduced to 0.91±0.43 after 1 week (p=0.028). Similarly, In the study by Eren et al., (2002), the plaque index was 0.73±0.44 before use of 15 ml of 0.12% chlorhexidine mouthrinse for 30 seconds twice daily, was reduced to 0.22± 0.23 after 2 weeks (p<0.001), thus substantiating antiplaque action of chlorhexidine.

Similarly, mean gingival index of patients in Group A (control group )was 1.98±0.60 as compared to 0.29±0.38 for those in the Group B (study group)[Table 4,Figure 15]. There was significant difference between gingival index of control and study groups (p<0.0001), thereby showing antigingivitis action of chlorhexidine.

The mean gingival index in study subgroups B1,B2, B3,B4 & B5 were 0.46±0.53, 0.45±0.41,0.06±0.05, 0.31±0.29, 0.18±.28 respectively with significant subgroup difference (p<0.001).

Like wise, Erdemir et al., (2007) in their study recorded gingival index of 1.66±0.13 prior 0.2% Chlorhexidine mouthrinse 10 ml twice daily use which was reduced to 1.17±0.17 after 1 week (p=0.018). Similarly, Eren et al.,(2002) in their study, the gingival index was 0.5±0.32 before use of 15 ml of 0.12% chlorhexidine mouthrinse for 30 seconds twice daily, was reduced to 0.13±0.18 after 2 weeks(p<0.0001). Thus, proving antiplaque action of chlorhexidine.

In our study genotoxicity was measured as number of micronucleated cells and number of micronuclei observed per 2000 buccal epithelial cells of the subject.

The mean number of micronucleated cells were found to be 0.41±0.71 in Group A (control group) as compared to 6.68±4.20 in Group B (study group), thus showing a statistically significant difference between two groups (p<0.001)[Table 5, Figure 16].
Similarly, the mean number of micronuclei was found to be 0.48±0.80 for Group A (control group) as compared to 8.70±5.15 for Group B (study group), thus showing a significant difference between two groups (p<0.001). [Table 5] These above findings thereby demonstrate genotoxic effects of CHX on buccal epithelial cells.

In a similar study on humans Erdemir et al., (2007) assessed the cytotoxicity of three commercial mouthrinses Klorhex (0.2% Chlorhexidine Gluconate), Andorex (0.15% Benzydamine HCL and 0.12% Chlorhexidine Gluconate) and Tanflex (0.15% Benzydamine HCL) on buccal epithelial cells using micronucleus (MN) test. Twenty eight patients aged 16-24 year undergone three mouthrinses’ application were analyzed before and after 1 week of exposure. Physiologic saline was used for the control group. The MN incidence was scored in the buccal epithelial of each participants. The difference in pre- and post-treatment after one week incidence of MN and plaque (PI) and gingival indices (GI) was compared by non-parametric statistical tests. The micronuclei incidence increased in Klorhex, Tanflex and Andorex groups after exposure to mouth rinses (P<.05). The findings of the study support the evidence of genotoxic effects of CHX mouthrinse on buccal epithelial cells.

In a more recent study on humans assessing chlorhexidine genotoxicity Carlin et al.,(2012) evaluated the comparative DNA damage and cellular death in buccal mucosa cells which were exposed to various commercially available five mouthrinses; Listerine® Cepacol®, Plax alcohol free®, Periogard®, and Plax Whitening®. Seventy five subjects were recruited in the study and were divided into five groups containing 15 people each and were exposed to respective commercial mouthrinse. Exfoliated buccal mucosa cells were taken before exposure and after 2 weeks of mouthrinse exposure. Also, blood was also taken from three healthy donors for in-vitro study. The single-cell gel(comet) assay was done to determine DNA damage done in-vitro where micronucleus test was done to evaluate the genotoxicity and cytotoxicity in vivo. The result showed that Periogard® (0.12% CHX) caused increase in Micronucleus frequency after 2 weeks of exposure with statistically significant difference (p<0.05) as compared to before. The genotoxic effect of CHX was further demonstrated by single-cell gel (comet) assay which showed DNA damage both in blood lymphocytes and buccal mucosa of individuals who were exposed to 0.12% chlorhexidine digluconate for 2 weeks.
Recently Arabaci et al.,(2013) in their study assessed the genetic and cellular toxicity of CHX on peripheral human lymphocytes in vitro. Micronucleus assay was used to investigate the genotoxicity, while cell viability and proliferation was evaluated by Trypan blue exclusion test and Nuclear Division Index in Control and CHX treated (0.05, 0.1, 0.2, 0.4, 0.5 mg/ml) human blood cultures. The result revealed a dose-dependent genotoxic and cytotoxic effects of CHX on human lymphocytes in vitro. This finding implicates the the prohibition of CHX as irrigating solution as in full mouth disinfection.

Also, genotoxic effect of chlorhexidine was assessed using Comet assay (Single cell gel electrophoresis) in human buccal epithelial cells and blood lymphocytes by study by Eren et al.,(2002), in which 13 subjects rinsed their mouth with 0.12% CHX solution for 18 days. Buccal epithelial cells and peripheral blood lymphocytes were obtained from all participants at baseline and at the end of experiment. A statistically significant amount of DNA damage in both buccal epithelial cells and blood lymphocytes was observed. The study demonstrated genotoxic effect of CHX on buccal epithelial cells and blood lymphocytes.

The genotoxic effect of chlorhexidine mouthwash was also shown in animal study on rats by Ribeiro et al.,(2004) who investigated the genotoxicity of chlorhexidine gluconate on rat peripheral blood and oromucosal cell by comet assay (single cell gel electrophoresis, or SCGE) and MN test. They took 30 wistar rats and divided them into three groups viz; Negative control, Experimental group were treated with 0.5 ml of 0.12% twice daily, for 8 days, Positive Control received 4-nitriquinoline 1-oxide at 0.5 gm/l by drinking water. A statistically significant increase in DNA damage was observed in oro mucosal cells and leukocytes of wistar rats who were exposed to chlorhexidine.

In a more rigorous study Grassi et al.,(2007) investigated the genotoxicity of CHX on leukocytes, liver, kidney and urinary bladder cells of wistar rats using single cell gel(comet) assay. Ten wistar were divided into two groups; 5 control and 5 experimental group treated with 3ml of 0.12% CHX by gavage once daily for 8 days. The rats were sacrificed at end of experiment and Blood, Liver and Kidney cells were taken for comet assay. A statistically significant DNA damage was seen in leukocytes and kidney cells.
The results of our study have refuted the null hypothesis which we stated in the beginning of the study that there is no difference in micronucleated cells and number of micronuclei in exfoliated buccal epithelial cells of chronic gingivitis patients exclusively on mechanical plaque control versus chronic gingivitis patients on mechanical plaque control measures with adjunct 0.2% Chlorhexidine mouthwash \( p<0.001 \). Thus demonstrating that chlorhexidine does have genotoxic effects on buccal epithelial cells of its users [Table 5, Figure 16].

Similar findings were shown by Carlin et al., (2012) who in their study observed 1.8% increase of micronucleated cells with significant statistical differences \( p<0.05 \) as compared to before \( (0.27\%) \) chlorhexidine mouthwash exposure for 2 weeks.

Also, Erdemir et al., (2007) in similar study showed increase in micronuclei from \( 5.57\pm5.00 \) before exposure of chlorhexidine mouthwash to \( 11.86\pm8.51 \) after exposure for 1 week.

Similarly, Eren et al., (2002) in their study using single cell gel electrophoresis (comet assay) showed significant DNA damage in buccal cells in subjects who rinsed their mouth with 15 ml of 0.12% CHX twice daily. There was significant \( (p<0.001) \) decrease in undamaged nuclei of buccal cells after CHX exposure from \( 91.54\pm6.75 \) to \( 71.15\pm7.12 \). However, there was significant \( (p<0.001) \) increase in intermediate nuclei from \( 6.00\pm4.85 \) before CHX exposure to \( 13.08\pm4.98 \) after exposure and also there was significant increase \( (p<0.001) \) in tailed nuclei from \( 2.46\pm3.73 \) before exposure to \( 15.77\pm4.6 \) after CHX exposure.

Also, Ribeirio et al., (2004) in their study on wistar rats treated with 0.5ml of 0.12% CHX digluconate twice daily for 8 days showed significant DNA damage in oral cells using single cell gel (comet) assay with tail movement of \( 1.34\pm0.46 \) as compared to \( 0.38\pm0.10 \) of negative control \( (p<0.05) \) and tail movement of \( 9.02\pm1.95 \) as compared to \( 3.47\pm0.58 \) of negative control \( (p<0.005) \).

Further, on comparing micronucleated cells in Group A \( (0.41\pm0.71) \) with subgroups of Group B; Groups B1, B2, B3, B4 and B5. The mean micronucleated cells in B1 \( (1.65\pm2.09) \), B2 \( (4.88\pm2.09) \), B3 \( (5.20\pm2.62) \), B4 \( (10.0\pm2.09) \) and B5 \( (11.7\pm1.87) \) showed an incremental trend with order of mean micronucleated cells in following sequence \( A < B1 < B2 \approx B3 < B4 < B5 \) \( (p<0.001) \). [Table 11, Figure 20]
Similarly, on comparing number of micronuclei in Group A (0.48±0.80) with subgroups of Group B. There was also an incremental trend in number of micronuclei cells seen from B1(2.57±1.64), B2(6.0±1.92), B3(6.69±2.21), B4(13.8±2.72) and B5(14.5±2.49) in the sequence of A < B1 < B2 ≤ B3 < B4 ≤ B5(p<0.001). [Table 11, Figure 20]

Our findings show that on increasing the duration of chlorhexidine use increases the total micronucleated cells and number of micronuclei in buccal epithelial cells of its users. This suggests that CHX causes cumulative genotoxicity and is dependent on time of exposure. Our observations have been substantiated by several studies viz; Lessa at al., (2010) in their invitro study exposed cultured odontoblast like cells(MDPC-23) to different concentrations of CHX(0.06%, 0.12%, 0.2%, 1% and 2%) for 60 s, 2 h or 60 s with 24 hr recovery period using MTT assay. They showed that CHX exhibited dose dependent toxic effect on odontoblasts like cells. The cytotoxic effect of CHX was more pronounced on increasing the contact time of CHX with cells.

Similarly, Gianelli et al.,(2008) in their invitro study exposed osteoblastic, endothelial and fibroblastic cell lines to various concentrations of CHX for different times and tested for cell viability and cell death. They found that CHX causes cell damage in concentration and time dependent manner.

Also, Bonacorsi et al., (2004) in their study evaluated CHX-induced cytotoxicity using MTS assay on murine peritoneal macrophages and showed that cytotoxicity of CHX is dependent on concentration and time of exposure.

Likewise, Hidalgo & Dominguez (2001) studied the mechanisms underlying CHX-induced toxicity on human fibroblasts using ATP–dependent Luciferin-Luciferase reaction using commercial ATP Bioluminescence Assay Kit (Boehringer Germany). Human fibroblasts were exposed to range of CHX concentrations of 0.00005–0.025% for 3, 6, 8 or 24 h in the absence or presence of different concentrations of foetal calf serum (FCS) (2, 5 and 10%). They observed that CHX causes time dependent cytotoxicity.

Chang et al.,(2001) also observed the cytotoxic effects of chlorhexidine on human periodontal ligament cells to be concentration and exposure time dependent.
The baseline frequencies for micronucleated cells in the buccal mucosa in the normal population ranges within 0.5–2.5 MN/1,000 cells (Holland et al., 2008), which is fairly equivalent to the number of micronucleated cells (0.41±0.71)/2000 cells in control (Group A) of our study.

The findings of our study suggest that CHX usage can only be justified after taking into account the risk to benefit ratio as CHX cause incremental increase in both micronucleated cells and total number of micronuclei leading to cumulative genotoxicity of CHX in buccal epithelial cells of its users.

Our study comprised of 356 subjects and the study was done in two parts; Clinical and Laboratory parts. The clinical part which consisted of recruiting the subject for the study, clinical examination and collection of buccal epithelial cells from the subjects. The collected sample were coded and sent to the laboratory for micronuclei assessment, thereby making the study single blind and thus reduced the bias.

Further our study is case-control and cross sectional in study design which does not give highest level of evidence whereas Erdemir et al (2007) assessed CHX genotoxicity in CHX interventional trial study with physiologic saline as control in which they compared MN scores before and after 1 week of CHX use.

Similarly Carlin et al., (2012) evaluated CHX genotoxicity in a interventional study in which they compared MN score before and after 2 weeks of chlorhexidine mouthrinse use. However our study gives an indication about the trend of genotoxicity on increasing the duration of chlorhexidine use ranging from 1 week to 24 weeks, which to our best of knowledge, is the first human study assessing the long term genotoxic effect of chlorhexidine use on buccal epithelial cells in clinical settings.

One more limitation of our study is that we relied on patient’s history and records for the duration and frequency of CHX use and did not employ any method to validate patient’s compliance of using CHX except recording the plaque and gingival indices. Also, we did not prospectively followed the patients for genotoxic assessment who took chlorhexidine mouthwash as adjunct mechanical plaque control measure.
Further, the Giemsa stain used for staining micronuclei in our study is a DNA non-specific stain and may give false positive results by staining cellular debris and artifacts and therefore may have exaggerated the effect (Holland et al., 2008).

Similarly, Erdemir et al., (2007) in their study using MN for CHX genotoxicity employed Giemsa stain similar to our study.

However, Carlin et al., (2012) using MN assay in buccal epithelial cells of chlorhexidine users used DNA specific Feulgen/ Fast Green method.

Although, methods and scoring data contribute 75% of total variance in micronuclei scoring and majority of variation had been attributed to laboratory methods (Bonsassi et al., 2001).

Also, Bonassi et al., (2001) observed that MN frequency is higher in females as compared to males owing to extra ‘x’ chromosome present in females is unstable can lead to more chromosomal breakage or loss and also there is steep increase in MN after 40 years of age which may be explained by cumulative exposure of environmental genotoxin and free radical injury incurred throughout the course of life.

Further, there were many unknown and undetected confounders which could influence micronucleus frequency such as nutritional status, Folic acid, vitamin B12 level, exposure to radiation, drugs, viral infections and other unknown genotoxic agents prevalent in the environment (Fenech & Bonassi, 2011; Suhas et al., 2004).

Despite the limitations, this study gives us an indication of potential genotoxic effects of chlorhexidine mouthwash on buccal epithelial cells and therefore it validates the claim for search of an antiplaque agent which has equal efficacy as chlorhexidine but without genotoxic effects and also warrants for formulation of guidelines for adjunctive use of chlorhexidine in clinical practice, considering its potential genotoxicity.

Therefore, further research with robust study designs such as longitudinal studies and randomized control trials in humans with DNA specific stains are required to elucidate the actual DNA damage in buccal epithelial cells of CHX users.
Our study reiterate, the importance of mechanical plaque control and also affirms the limited and restricted use of adjunct chlorhexidine, which is a potential genotoxic agent.

Therefore, patients who fail to maintain adequate plaque control exclusively on mechanical plaque control measures should only be prescribed chemical plaque control agents as an adjunct to mechanical plaque control under professional supervision.

Patients should be educated and motivated for maintaining adequate plaque control by demonstration of proper tooth brushing technique and use of interdental plaque control aids such as dental floss & interdental brushes by the dental professional.

The mechanical plaque control dexterity of the patient can be improved by regular motivation, education, demonstration and re-evaluation by the dental professionals, this will lead to less dependence on adjunct chemical plaque control methods.

The patients should be discouraged and cautioned of using over the counter oral hygiene products such as mouthrinses, sprays etc as self-medications without professional monitoring.

Our study also gives impetus for formulation of guidelines of chlorhexidine mouthrinse in clinical practice considering its potential cytotoxic and genotoxic effects on oral cells.