INTRODUCTION

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The objectives of root canal treatment include the removal of infected pulp tissue, elimination of bacteria or fungi present in the canal as well as dentinal tubules and prevention of recontamination after treatment. These objectives are achieved by thorough cleaning, shaping, and disinfecting the root canal system as well as sealing it with a 3-dimensional obturation and by placing a coronal seal.[1]

The extraordinary complex root canal system has allowed researchers in the past few decades to extrapolate its complexities through the intervention of new technologies which have revealed the real complex nature of the root canal system which extend far beyond the reach of hand and mechanically driven endodontic instruments. Such root canal intricacies are resistant or inaccessible even to intracanal irrigants and medicaments. Available literature from previous studies have reported that almost 35% of the canal walls remain completely untouched regardless of the method of biomechanical preparation or the various file systems used. Moreover, mechanical instrumentation usually results in an amorphous irregular smear layer composed of inorganic and organic material covering the canal surfaces and plugging the dentinal tubules.[2] Therefore, the mechanical instrumentation needs to be augmented with thorough intracanal irrigation with an effective irrigant to best disinfect such shrouded intricacies.

Irrigation is an unavoidable, essential component of root canal preparation. The purpose of irrigation during root canal preparation includes wetting of the root canal walls, flushing out of debris, destruction of microorganisms, dissolution of organic matter and softening of dentin to remove smear layer. Any further disinfection of the root canal system will only occur with the support of an intracanal dressing.[2]

Till date, Sodium hypochlorite solution is the most employed root canal irrigant since the time Walker introduced it into the field of endodontics in 1936.[3] This is mainly due to its unique capacity to dissolve organic matter[4], neutralize and degrade fatty acids and amino acids as well as disrupt the biofilm.[3] The effectiveness of organic

tissue dissolution by NaOCl is well known.[4-10] Although it is beneficial during root canal treatment, it is a hazard for normal tissue if it comes in contact with it. In addition, NaOCl is chemically unstable and external agents such as temperature, light and storage conditions can influence the availability of chlorine ions and interfere with its effectiveness.[4,10] Therefore, alternative auxiliary chemical solutions should be investigated.

One such promising endodontic irrigant is found to be $Ca(OCl)_2$. It has shown comparable results with NaOCl in terms of tissue dissolution and antimicrobial activity. It has higher chlorine content than NaOCl at the same concentration.[11] In endodontics, the main outcome of the treatment is the periapical and apical tissue repair. The chemical solutions used during therapy can accelerate or retard the healing process. In this regard, $Ca(OCl)_2$ showed more satisfactory results than NaOCl.[12] However, there is a lack of consistent information regarding the properties of $Ca(OCl)_2$ with reference to toxicity in comparison to NaOCl which is cytotoxic in high concentrations.[13]

Genotoxicity tests are often defined as in vitro and in vivo assays designed to detect compounds that induce genetic damage including DNA damage, point mutation, chromosomal breakage, altered DNA repair capacity and cellular transformation. It has been postulated that exposure of living tissues to cytotoxic agents may result in chronic cell injury, compensatory cell proliferation, hyperplasia, irritation, degeneration, or tissue necrosis[14] and ultimately tumour development.[14,15] It is likely that proliferation may increase the risk of mutations within the target cells and be important in selective clonal expansion of exogenously or endogenously initiated cells from pre-neoplastic foci and eventually tumours.[15] Thus, the DNA damage may diminish the self-repairing potential of the tissue.

Cytotoxicity is one among the foremost important indicators for biological evaluation of in vitro studies. In vitro, chemicals have different cytotoxicity mechanisms like destruction of cell membranes, prevention of protein synthesis, irreversible binding to

receptors etc. To determine the cell death caused by these damages, there is a need for reliable and reproducible short-term cytotoxicity assays.

Considering that there is a high chance for the extrusion of irrigants beyond the apical constriction, resulting in direct contact with the periapical tissue[16],it is important to determine the genotoxicity and cytotoxicity assays which have gained widespread acceptance as a crucial and useful indicator for carcinogenicity.

Thus, the aim of the present study is to Evaluate the Cytotoxicity and Genotoxicity of 2.5% and 5.25% Calcium Hypochlorite in comparison with 2.5% and 5.25% Sodium Hypochlorite, *In Vitro.*

AIM & OBJECTIVES

AIM & OBJECTIVES

AIM

To Evaluate the Cytotoxicity and Genotoxicity of 2.5% and 5.25% Calcium hypochlorite in comparison with 2.5% and 5.25% Sodium hypochlorite: An In Vitro Study

OBJECTIVES

- To evaluate the Cytotoxicity of 2.5% and 5.25% Calcium hypochlorite in comparison with 2.5% and 5.25% Sodium hypochlorite by checking the presence of non-viable cells using Trypan blue Assay.
- To evaluate the Genotoxicity of 2.5% and 5.25% Calcium hypochlorite in comparison with 2.5% and 5.25% Sodium hypochlorite by checking the presence of micronuclei using Cytokinesis block micronucleus Assay.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

- **R. Kakehashi et al. (1965)**[17] in their in vivo study of the effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats reported that the presence or absence of a microbial flora is the major determinant in the healing of exposed rodent pulps.[17]

- **Spangberg and Langeland et al. (1973)**[18] carried out a series of *in vivo* and *in vitro* tests on various potential irrigants. They found that in addition to being highly toxic and irritating, 5% sodium hypochlorite was considerably stronger than necessary to kill the bacteria in the root canal, while 0.5% concentration dissolves necrotic tissue but has no effect on *Staphylococcus aureus*.[18]

- **Harrison and Hand et al. (1981)**[19] showed that the dilution of 5.25% NaOCl resulted in a significant decrease in its ability to dissolve necrotic tissue. In this investigation, examination through scanning electron microscope of un-instrumented surfaces could not detect any difference in the removal of pulpal remnants and pre-dentin in the middle third of the root canals with 5.25%, 2.5%, and 1% NaOCl delivered with either a needle or an ultrasonic device. [19]

- **W R Moorer et al. (1982)**[20] reported that the tissue dissolving capacity of NaOCl solution depended on the frequency and intensity of mechanical agitation as well as the surface area available for the free or enclosed tissue.[20]

- **Russell S. Yamada et al. (1983)** [21] in their literature on scanning electron microscopic comparison of high-volume final flush with several irrigating solutions concluded that the final flush with 10 ml of 17% EDTA buffered to pH 7.7 followed by 10 ml of 5.25% NaOCl solution was the most effective to clean the root canal after the completion of instrumentation.[21]

- **Byström A et al. (1985)**[22] evaluated the antibacterial effect of irrigating infected root canals with 0.5% and 5% sodium hypochlorite solutions clinically.

The results indicated that there was no difference between the antibacterial effect of those two solutions. The combined use of EDTA and 5% sodium hypochlorite solution was more efficient than the use of sodium hypochlorite solutions alone. Also, bacteria surviving instrumentation and irrigation rapidly increased in number in the period between appointments when no intracanal medicament was used.[22]

- **Kaufman A Y et al. (1989)**[23] reported a case in which hypersensitivity to household bleach was proved with skin patch tests. The clinician was warned of the possible health hazard from using NaOCl in this patient by the past medical history. Endodontic therapy was carried out with an irrigant not containing NaOCl after the allergy to NaOCl was verified. Treatment was uneventful. It is suggested that before any endodontic treatment in which sodium hypochlorite is to be used, the patient should be asked about hypersensitivity to household bleaching materials.[23]

- **Ciucchi et al. (1989)**[24] found that after ultrasonic irrigation of root canals for 2 min with 3% NaOC1, 27% of the observed surfaces were smear free in the middle third. In this study, the smear layer on the instrumented portion of the root canals appeared to be very similar irrespective of the concentration of the NaOCl. Exposed dentinal tubules could be seen in the smear layer irrespective of whether the irrigant was delivered by needle or ultrasonic device.[24]

- **Baumgartner JC et al. (1992)**[25] used scanning electron microscope to examine the instrumented and uninstrumented surfaces in the middle third of the root canal following the use of several concentrations of NaOCl (5.25%, 2.5%, 1.0%, and 0.5%). Pulpal remnants and predentin from the uninstrumented surfaces were completely removed. Although 0.5% NaOCl removed majority of the pulpal remnants and predentin from the uninstrumented surfaces, it left some fibrils on the surface.[25]

- **Johnson B R et al. (1993)**[26] investigated the variables of storage conditions and time on the tissue dissolving capacity of three different concentrations of

sodium hypochlorite. The tissue dissolution of 5.25% sodium hypochlorite remained stable for at least 10 weeks. The tissue-dissolving ability of 2.62% and 1.0% NaOCl remained relatively stable for 1 week after mixing, then exhibited a significant decrease in the tissue dissolving ability at 2 weeks and beyond.[26]

- **David R. Drake et al. (1994)**[27] in his in vitro study on bacterial retention in canal walls on the effect of smear layer concluded that smear layer produced during root canal therapy inhibited bacterial colonization of root canals.[27]

- **Piskin D et al. (1995)**[28] investigated the effects of storage temperature, concentration, and time on the stability on three different brands of commercial household bleaching agents as a source of NaOCl, and compared the stability of these brands. All solutions showed degradation versus time; however, this degradation occurred very slowly except for the group of solutions containing 5% available chlorine stored at 24°C. Solutions containing 0.5% available chlorine stored at 4°C and 24°C and 5% solutions stored at 4°C showed satisfactory stability at 200 days.[28]

- **Hulsmann H et al. (2000)**[29] reported that the tissue dissolving ability of NaOCl is directly related to its concentration, which is also related to its ability to irritate the periapical tissues.[29]

- **H. Dwight Moss et al. (2001)**[30] in his survey on philosophies and practices regarding the management of the endodontic smear layer proposed that there is no clear consensus in the endodontic community, either academically or clinically, as to whether the smear layer should be removed or be allowed to remain before obturation of the root canal space.[30]

- **Vianna M E et al. (2004)**[31] investigated in vitro the antimicrobial activity of 0.2%, 1%, and 2% chlorhexidine gluconate (CHX gel and CHX liquid), against endodontic pathogens and compared the results with the ones achieved by 0.5%, 1%, 2.5%, 4%, and 5.25% sodium hypochlorite (NaOCl). The timing required

for 1.0% and 2.0% CHX liquid to eliminate all microorganisms was the same as required by 5.25% NaOCl.[31]

- **Brent J. Crumpton et al. (2005)**[32] in their study of effects on smear layer and debris removal with varying volumes of 17% REDTA after rotary instrumentation concluded that the efficient removal of smear layer was accomplished with a final rinse of 1 ml of 17% EDTA for 1 min, followed by 3 ml of 5.25% NaOCl.[32]

- An SEM analysis by **C. S. Teixeira et al. (2005)**[33] on the effect of application time of EDTA and NaOCl on intracanal smear layer removal concluded that canal irrigation with EDTA and NaOCl for 1, 3 and 5 min were equally effective in removing the smear layer from the canal walls of straight roots.[33]

- **Berber et al. (2006)**[34] evaluated the efficacy of 0.5%, 2.5% and 5.25% sodium hypochlorite (NaOCl) as intracanal irrigants against Enterococcus faecalis within root canals and dentinal tubule associated with hand and rotary instrumentation techniques. They found that 5.25% NaOCl was shown to be the most effective irrigant solution tested, when dentinal tubules were analysed at all depths of the root canals and by all techniques used, followed by 2.5% NaOCl. No difference was found among the various concentrations in cleaning the canals.[34]

- **Khademi et al. (2006)**[35] in an in vitro study compared the antimicrobial substantivity of 2% chlorhexidine gluconate (CHX), 100 mg/ml Doxycycline and 2.6% Sodium hypochlorite (NaOCl) in bovine root dentine. They found that in the first culture, the NaOCl group and doxycycline HCl group showed the lowest and highest number of Colony Forming Units (CFU), respectively. In each group, the number of CFU increased significantly by time lapse ($P < 0.05$). In conclusion, the substantivity of CHX was significantly greater than NaOCl and Doxycycline.[35]

- **Olivera et al. (2007)**[36] conducted a study to compare the in vitro antimicrobial activity of 2% chlorhexidine gel against Enterococcus faecalis with sodium hypochlorite in 2 different concentrations (1.5% and 5.25%). The microbiological samples were plated to count the colony forming units (CFU). They concluded that 2% chlorhexidine gluconate gel and 5.25% sodium hypochlorite were effective in eliminating E. faecalis 7 days after instrumentation. Moreover, higher the concentration of sodium hypochlorite better was its antimicrobial action.[36]

- **Deborah Clark-Holke et al. (2012)**[37] in his study on bacterial penetration through canals of endodontically treated teeth in the presence or absence of the smear layer proposed that removal of smear layer reduced the leakage of bacteria through the root canal.[37]

- **Dutta A et al. (2012)**[38] compared *in vitro* the tissue-dissolution properties of 5% and 10% calcium hypochlorite ($Ca(OCl)_2$) with two concentrations (1.36% and 4.65%) of proprietary sodium hypochlorite (NaOCl) on bovine muscle tissue. Chlorax (4.65% NaOCl) dissolved tissue faster than the $Ca(OCl)_2$ solutions and Tesco thin bleach (1.36% NaOCl) over the first 35 minutes, but there was no significant difference among the solutions thereafter.[38]

- **Silveira LF et al. (2013)**[39] in their analysis by scanning electron microscope concluded that the cleaning efficacy of a 2.5% sodium hypochlorite (NaOCl) and a 17% EDTA solution either applied alternately or mixed together for smear layer removal during root canal preparation has been shown to be the most effective form of irrigation in the removal of smear layer.[39]

- **Tirali et al. (2013)**[40] compared the antimicrobial activity of sodium hypochlorite (NaOCl), chlorhexidine gluconate (CHX) and octenidine hydrochloride (OCT) in different concentrations against endodontic pathogens in vitro. 5.25% NaOCl exhibited better antimicrobial effect than the other

concentrations of NaOCl for all strains. Decreasing the concentrations of NaOCl resulted in significantly reduced antimicrobial effect.[40]

- **Hegde et al. (2013)**[41] conducted a study to assess the antimicrobial activity of 2% sodium hypochlorite, propolis, neem leaf extract, turmeric, and liquorice against E. Faecalis and C. Albicans using the agar diffusion method. They concluded that Sodium hypochlorite remained the gold standard for irrigation in primary endodontic infections.[41]

- **de Almeida AP et al. (2014)**[42] compared *in vitro* the effectiveness of calcium hypochlorite ($Ca[OCl]_2$) and sodium hypochlorite (NaOCl) associated with passive ultrasonic irrigation in root canals of bovine teeth infected with *Enterococcus faecalis*. $Ca(OCl)_2$ as well as passive ultrasonic irrigation aided in chemo-mechanical preparation, contributing in a significant way to the reduction of microbial content during root canal treatment.[42]

- **Taneja S et al. (2014)**[43] compared human pulp tissue dissolution by different concentrations of chlorine dioxide, calcium hypochlorite and sodium hypochlorite and reported that NaOCl most efficiently dissolved the pulp tissue at both concentrations and at both time intervals. Mean tissue dissolution by $Ca(OCl)_2$ and ClO_2 gradually increased with time and with increase in concentration.[43]

- **Dumani A et al. (2015)**[44] compared the in vitro efficacy of calcium hypochlorite ($Ca(OCl)_2$) and sodium hypochlorite (NaOCl) associated with sonic (Vibringe) irrigation system in root canals which were contaminated with *Enterococcus faecalis*. The antimicrobial property of $Ca(OCl)_2$ has been investigated and compared with that of NaOCl. Both conventional syringe irrigation and sonic irrigation were found to be effective in removing *E. faecalis* from the root canal of extracted human teeth.[44]

- **Shahadeh et al. (2015)**[45] evaluated the ability of a new irrigant QMix, in the microbial reduction of Enterococcus faecalis from infected root canals in

comparison with sodium hypochlorite 5.25%. The bacterial swabs were taken by sterilized paper points in two stages; the first one was after the end of incubation period, and the second one was immediately after the irrigation. They concluded that sodium hypochlorite 5.25%, had clear ability on the microbial reduction of E. faecalis in comparison with the new irrigant QMix.[45]

- **Carlotto IB et al. (2016)**[46] evaluated the pH and the available chlorine content from sodium hypochlorite (NaOCl) and calcium hypochlorite ($Ca(OCl)_2$) solutions stored in different conditions and time periods as well as the surface tension of $Ca(OCl)_2$ solutions in comparison with NaOCl. $Ca(OCl)_2$ solutions are extremely alkaline and tend to have more available chlorine content than NaOCl but have a higher surface tension than NaOCl. Regarding the available chlorine content, these solutions tend to be stable for 30 days of storage when kept at 4°C or at 25°C.[46]

- **Blattes et al. (2017)**[47] analysed cytotoxicity of calcium hypochlorite ($Ca(OCl)_2$) solutions in comparison with sodium hypochlorite (NaOCl) solutions on cultured 3T3 fibroblasts in vitro and inflammatory reaction in rats in vivo. $Ca(OCl)_2$ showed favourable results of viability and induced a low-level inflammatory response. $Ca(OCl)_2$ presented acceptable cytotoxicity and biocompatibility as an irrigant solution.[47]

- **Duvvi SAB et al. (2018)**[48] reported that 2.5% NaOCl and 5% $Ca(OCl)_2$ showed less reduction in microhardness of root canal dentin when compared with 5% NaOCl and 10% $Ca(OCl)_2$.[48]

- **G. Faria et al. (2018)**[49] NaOCl gel penetrated less into dentinal tubules than NaOCl solution. The addition of surfactants did not increase the penetration depth. The use of PUI significantly increased NaOCl penetration into dentinal tubules.[49]

- **Iglesias JE et al. (2019)**[50] reported that the addition of surfactant to 2.5% $Ca(OCl)_2$ showed acceptable outcome for pH, free chlorine content, surface

tension, contact angle, pulp dissolution and antimicrobial activity. Also, the addition of 0.2% Cetrimide showed better results for all tested properties.[50]

- **Coaguila-Llerena H et al. (2019)**[51] assessed cytotoxicity and cell migration of calcium hypochlorite [$Ca(OCl)_2$] and octenidine hydrochloride(OCT). Cell migration of 0.1% OCT, 2% CHX and 2.5% $Ca(OCl)_2$ groups was higher than 5% $Ca(OCl)_2$ and 2.5% NaOCl groups at 24 h.[51]

- **Shadmehr E et al. (2019)**[52] concluded that 5% Calcium hypochlorite and 2% Chlorhexidine were more effective than 5.25% NaOCl in the reduction of mixed culture biofilm.[52]

- **Paula KB et al. (2019)**[53] compared the antimicrobial activity and tissue dissolution capacity of calcium hypochlorite ($Ca(OCl)_2$) solution with sodium hypochlorite (NaOCl) solution at 0.5%, 1.0%, 2.5%, and 5.25% concentrations. $Ca(OCl)_2$ solutions showed antimicrobial activity against *E. faecalis* and can dissolve pulp tissues.[53]

- **Kaur G et al. (2020)**[54] reported that 5% $Ca(OCl)_2$ solution with 1% Chitosan Oligosaccharide solution effectively removed the *E. faecalis* biofilm and smear layer from the root canals with minimal erosion.[54]

- **Souza MA et al. (2020)**[55] evaluated the antimicrobial action of calcium hypochlorite ($Ca(OCl)_2$) and sodium hypochlorite (NaOCl) with reciprocating instrumentation and photodynamic therapy (PDT), and its influence on root dentin structure. The association of $Ca(OCl)_2$, reciprocating instrumentation and PDT promotes effective antimicrobial action. Also, lower modification was induced in microhardness and organic components of root dentin, by using $Ca(OCl)_2$ in low concentration associated to PDT.[55]

- **Durigon M et al. (2020)**[56] reported that calcium hypochlorite and grape seed extract kept the mechanical properties of root dentin but did not improve the fracture resistance of weakened roots.[56]

- **Yilmaz S et al. (2020)**[57] concluded that both, NaOCl and $Ca(OCl)_2$ solutions were cytotoxic and genotoxic to NIH3T3. However, $Ca(OCl)_2$ always had a significantly higher damaged cell percentage than NaOCl when investigated.[57]

- **Jose J et al. (2021)**[58] reported that Calcium hypochlorite is less cytotoxic than NaOCl, and when used in combination with EDTA, it was shown to have reduced cytotoxic effect on hGF cells to a great extent.[58]

- **Ghahderijani MS et al. (2021)**[59] compared the effect of $Ca(OCl)_2$ and NaOCl as root canal irrigants on the push out bond strength of fibre posts cemented with an etch and rinse resin cement. $Ca(OCl)_2$ decreased the push out bond strength of fibre posts cemented with an etch and rinse resin cement.[59]

RELEVANCE

RELEVANCE

The success of endodontic therapy mainly depends upon the proper cleaning of the root canal space. Instrumentation cannot solely remove all the debris and contaminants. This highlights the importance of chemical cleaning and disinfection of the root canal system. Mechanical instrumentation usually leads to the formation of an amorphous irregular smear layer which is composed of inorganic and organic material that covers the canal surfaces and plugs the dentinal tubules.

Sodium hypochlorite (NaOCl), neutralizes and degrades fatty acids and amino acids. It is the most commonly used irrigation solution. The effectiveness of organic tissue dissolution by NaOCl is well known and so even if its beneficial during root canal treatment, it is a hazard to the normal tissue if it comes in contact with it. In addition, NaOCl is chemically unstable and external agents, like temperature, light and storage conditions can influence the availability of chlorine ions and interfere with its effectiveness. Therefore, other auxiliary chemical solutions should be investigated.

In endodontics, a lack of consistent information exists regarding the properties of $Ca(OCl)_2$ solution as an irrigant for the root canal system. $Ca(OCl)_2$ showed comparable results to NaOCl in terms of tissue dissolution and antimicrobial activity. But NaOCl is cytotoxic at high concentrations. Thus, the aim of the present study was to evaluate the cytotoxicity and genotoxicity of 2.5% and 5.25% calcium hypochlorite in comparison with 2.5% and 5.25% sodium hypochlorite *in vitro.*

MATERIALS AND METHODS

MATERIALS AND METHODS

RESEARCH APPROACH

Qualitative and Quantitative analysis

RESEARCH HYPOTHESIS

There is significant difference in the cytotoxicity and genotoxicity of 2.5% and 5.25% Calcium hypochlorite and 2.5% and 5.25% Sodium hypochlorite.

NULL HYPOTHESIS

The null hypothesis is that there would be no difference in the cytotoxicity and genotoxicity of the solutions on the cells tested.

STUDY DESIGN

In vitro study

STUDY SETTING

Study was conducted at:

- St. Gregorios Dental College, Chelad, Kothamangalam.
- Sri Ramachandra University & Hospital, Chennai.
- Athmic Biotech Solutions Pvt. Ltd, Thiruvananthapuram.

SAMPLE AND SAMPLE SIZE

Sample size was calculated using statistical package G*power (3.1.5)

Minimum total sample size required for this study was 10.

SAMPLING PROCEDURE

About 2-3 ml of peripheral blood was collected from 10 study subjects.

An Informed Consent was taken from the subjects prior to sample collection.

Ethical clearance was obtained for the conduction of the study.

INCLUSION CRITERIA

Subjects above 18 years, without any history of smoking, alcohol consumption and who were willing to give their consent for observation and experimentation of their blood sample.

EXCLUSION CRITERIA

Subjects with co-morbidities like diabetes, hypertension, or cardiovascular disease and who were unwilling to provide consent for observing and experimenting their blood sample.

ARMAMENTARIUM

Materials

Calcium hypochlorite powder

Distilled water

Sodium hypochlorite

Roswell Park Memorial Institute medium (RPMI-Himedia)

10% heat inactivated Foetal Bovine Serum (FBS)

1% antibiotic cocktail containing Penicillin (100U/ml)

Streptomycin (100µg/ml)

Amphotericin B (2.5µg/ml)

Phytohemagglutinin (20 μg/ml)

Colchicine

Cytochalasin B

0.075 M KCl hypotonic solution

Phosphate Buffered Saline

Methanol

Glacial acetic acid

5% Giemsa stain

Xylene

Canadian balsam

Trypan Blue solution

TC flasks

Bottles

Millipore filter

Test tubes

Equipments

Cell culture incubator (Galaxy® 170 Eppendorf, Germany)

Centrifuge

Inverted phase contrast microscope

Light microscope

Hemocytometer

MATERIALS USED FOR THE STUDY

Calcium hypochlorite powder was made into a solution for use, by dissolving in distilled water to reach the two target concentrations of 2.5% and 5.25%.

Figure 1 a: Calcium Hypochlorite Powder

Figure 1 b: Calcium Hypochlorite solution in different concentrations

Figure 2: Different Concentrations of Sodium Hypochlorite

Preparation of the Solutions:

- The Solutions were prepared immediately before the experiments, as described by Blattes et al.[47]
- A 12% NaOCl solution was diluted using sterilized and distilled water to obtain the two target concentrations of 2.5% and 5.25%.
- $Ca(OCl)_2$ powder with 65% purity was dissolved in distilled and sterilized water to obtain the two target concentrations of 2.5% and 5.25%.
- After total dissolution, the solutions were filtered twice with millipore filtration to remove debris and stored in blinded, randomly numbered bottles.
- Human peripheral blood cells were used as the test system. Peripheral venous blood was collected from healthy volunteers. Informed consent was obtained from the donors at the time of donation for the use of their blood sample in this study.

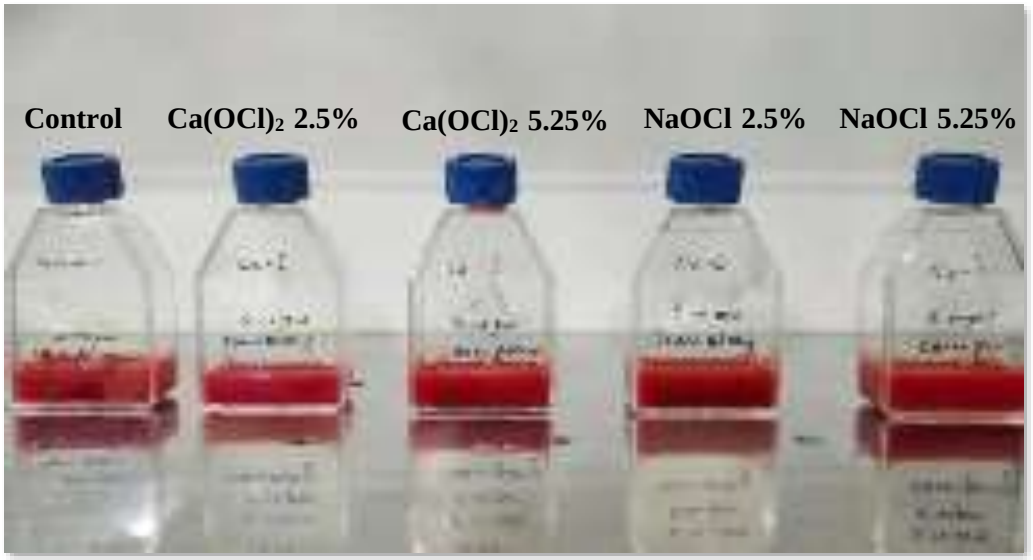


Figure 3: Blood Samples

The Groups were assigned as follows:

GROUP 1 (G1) : 2.5% Sodium hypochlorite solution and culture media

GROUP 2 (G2) : 5.25% Sodium hypochlorite solution and culture media.

GROUP 3 (G3) : 2.5% Calcium hypochlorite solution and culture media.

GROUP 4 (G4) : 5.25% Calcium hypochlorite solution and culture media.

GROUP 5 (G5) : Control group with culture media only.

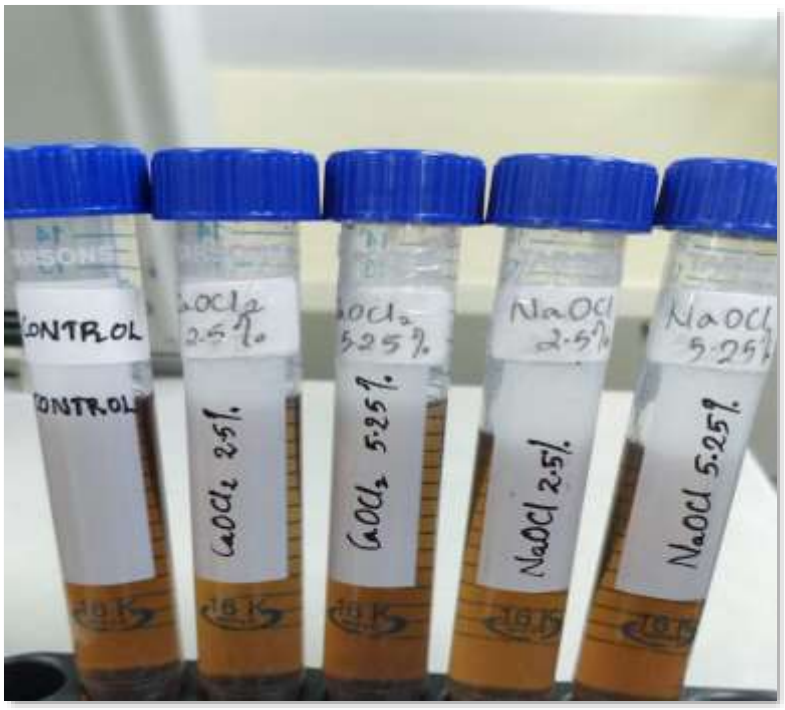


Figure 4: Test Solutions in Culture Medium

Cytokinesis-block micronucleus (CBMN) Assay for assessing Genotoxicity

Cell culture media and maintenance

The peripheral lymphocytes were cultured in Roswell Park Memorial Institute medium (RPMI-Himedia), supplemented with 10% heat inactivated Foetal Bovine Serum (FBS) and a 1% antibiotic cocktail containing Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). The cell containing TC flasks ($25cm^2$) were incubated at 37^OC at 5% CO_2 environment with humidity, in a cell culture incubator (Galaxy® 170 Eppendorf, Germany).

Assay Protocol

CBMN was carried out according to the protocol of Fenech and OECD guidelines.[60,61] About 400 µl of phytohemagglutinin (20 µg/ml) was used to initiate the culture. The

cells were seeded in each flask with 2.5 x 10^5 cells/flask, and after 24 hours of culture, the cells were exposed to different concentrations (10 µg/ ml, 25 µg/ml, 50 µg/ml, 100 µg/ml) of Calcium hypochlorite and Sodium hypochlorite for 48 hours.

Figure 5: Phytohemagglutinin

Figure 6: Cytochalasin B

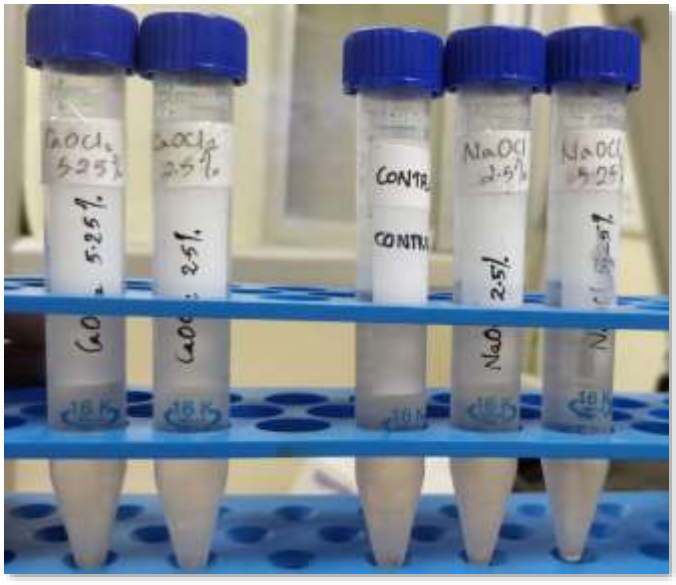


Figure 7: Test Solutions after adding Cytochalasin B

Colchicine was used as a positive control at 5 µg/ml at the same time of treatment. Cytochalasin B was added no later than 20 hours to the cell cultures at a final concentration of 3 µg/ml. The cells were blocked at cytokinesis by adding Cytochalasin B. Cells were harvested after an additional 24 hours and centrifuged for 8 minutes at 1100 rpm. The supernatant was removed, and the cells were treated for 1 min with 0.075 M KCl hypotonic solution.

Figure 8: Centrifuge

The cells were then washed in PBS, resuspended (approximately 5 x 10^6 cells/ml) and spread onto the glass slide (20 µl of cell suspension per slide). After air-drying, the cells were fixed twice with methanol and glacial acetic acid in the ratio of 6:1 for 10 min and stained with 5% Giemsa solution for 5 min. All procedures were conducted at room temperature. After washing with distilled water, the slides were rapidly dried in xylene and mounted with Canadian balsam.

OUTCOME MEASUREMENT

For each sample, 1000 lymphocytes were checked and binucleated cells were scored using a light microscope with inverted phase contrast (40X magnification).

Trypan Blue Exclusion Assay

Principle:

The dye exclusion test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that cells that are living possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension was mixed with the dye and then visually examined to determine whether the cells took up or excluded the dye. In the protocol presented here, a viable cell would have a brighter appearance whereas a nonviable cell would have a dark blue appearance.

Assay Procedure:

1. An aliquot of cell suspension was tested for viability for 5 min at 100 Å~ g, was centrifuged and the supernatant was discarded.

   The size of the aliquot depended on the approximate number of cells present. The aliquot contained an adequate number of cells to count in a hemocytometer when suspended in 1 ml PBS. It was diluted again by mixing with 0.4% trypan blue.

Figure 9: Trypan Blue

2. The cell pellet was resuspended in 1 ml PBS or serum-free complete medium.

   Since Serum proteins stained with trypan blue can produce misleading results, the determinations were made in a serum-free solution.

3. 1 part of 0.4% trypan blue and 1 part cell suspension (dilution of cells) was mixed. The mixture was allowed to incubate ~3 min at room temperature.

   The cells were counted within 3 to 5 min of mixing with trypan blue, as longer incubation periods would lead to cell death and reduced viability counts.

   Mixing was performed in the well of a microtiter plate or a small plastic test tube using 10 to 20 µl each of cell suspension and trypan blue.

4. A drop of the trypan blue/cell mixture was added to the hemocytometer. The hemocytometer was placed on the stage of a binocular microscope and focussed on the cells.

Figure 10: Hemocytometer

OUTCOME MEASUREMENT

The unstained (viable) and stained (nonviable) cells were counted separately in the hemocytometer. To obtain the total number of viable cells per ml of aliquot, the total number of viable cells were multiplied by 2 (the dilution factor for trypan blue). To obtain the total number of cells per ml of aliquot, the total number of viable and nonviable cells were added and then multiplied by 2.

The percentage of viable cells were calculated as follows:

$$\textbf{Viable cells (\%)} = \frac{\textbf{Total number of viable cells per ml of aliquot}}{\textbf{Total number of cells per ml of aliquot}} \textbf{ X } \textbf{100}$$

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

The data recorded on the computer was collected, tabulated, and statistically analyzed. One way ANOVA test was done for the analysis of differences between the groups and then the post hoc analysis was done to find out the significant difference between any of the two given groups. Statistical analyses were performed using SPSS software (IBM). In all the analysis, significance level was taken to be 0.05 (i.e., if the p-value is less than 0.05, the null hypothesis would be rejected or it can be concluded that the null hypothesis is statistically significant).

CBMN Assay

Table 1: Mean and Standard deviation

Mean and Standard deviation											
CBMN Assay											
						95% Confidence Interval for Mean					
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	df	Mean Square	F	P Value
Binuclei Percentage	G1	10	16.1	2.33095	0.73711	14.4325	17.7675	3	1291.758	427.813	0.00
	G2	10	33.9	1.91195	0.60461	32.5323	35.2677				
	G3	10	7.5	1.17851	0.37268	6.6569	8.3431				
	G4	10	13.4	1.26491	0.4	12.4951	14.3049				
Total		40	17.7	10.10709	1.59807	14.4926	20.9574				
Multi nuclei Percentage	G1	10	23.9	2.18327	0.69041	22.3382	25.4618	3	1615.692	380.910	0.00
	G2	10	43.5	1.58114	0.5	42.3689	44.6311				
	G3	10	12.9	1.66333	0.52599	11.7101	14.0899				
	G4	10	24.4	2.63312	0.83267	22.5164	26.2836				
Total		40	26.17	11.32252	1.79025	22.5539	29.7961				
Total Micronuclei Percentage	G1	10	40.0	2.53859	0.80277	38.184	41.816	3	5756.4	1034.000	0.00
	G2	10	77.4	1.26491	0.4	76.4951	78.3049				
	G3	10	20.4	2.27058	0.71802	18.7757	22.0243				
	G4	10	37.8	3.01109	0.95219	35.6460	39.9540				
Total		40	43.9	21.16456	3.34641	37.1312	50.6688				

Graph 1: Mean and Standard deviation (Binuclei Percentage)

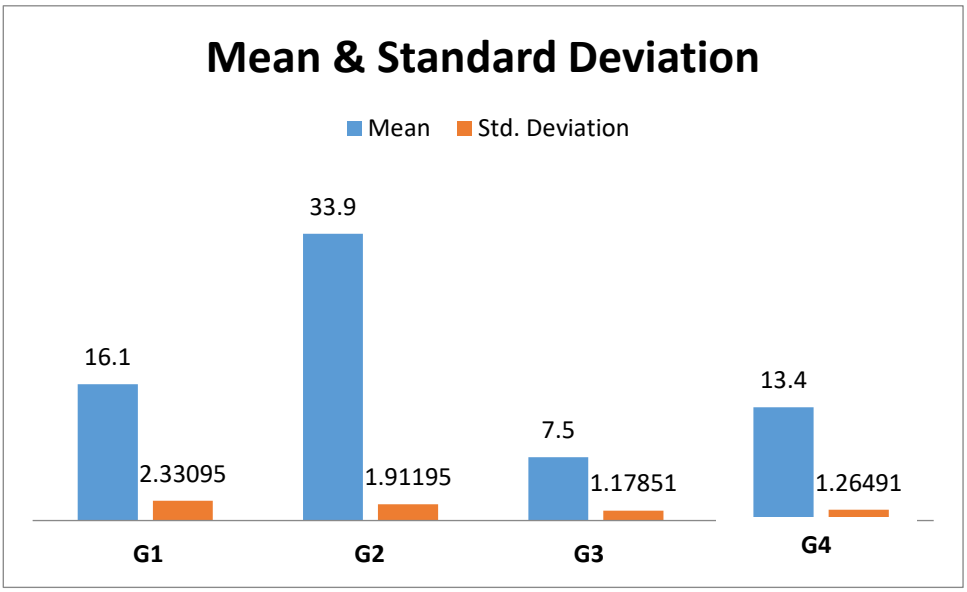
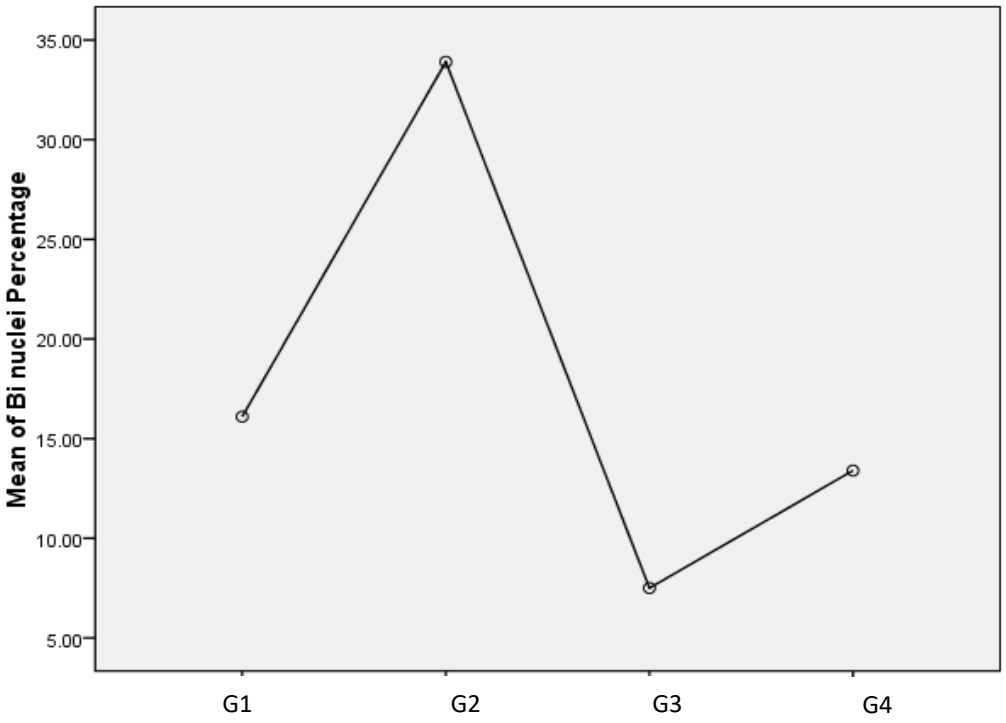


Figure 11: Diagramatic Representation of Binuclei Percentage



Graph 2: Mean and Standard deviation (Multinuclei Percentage)

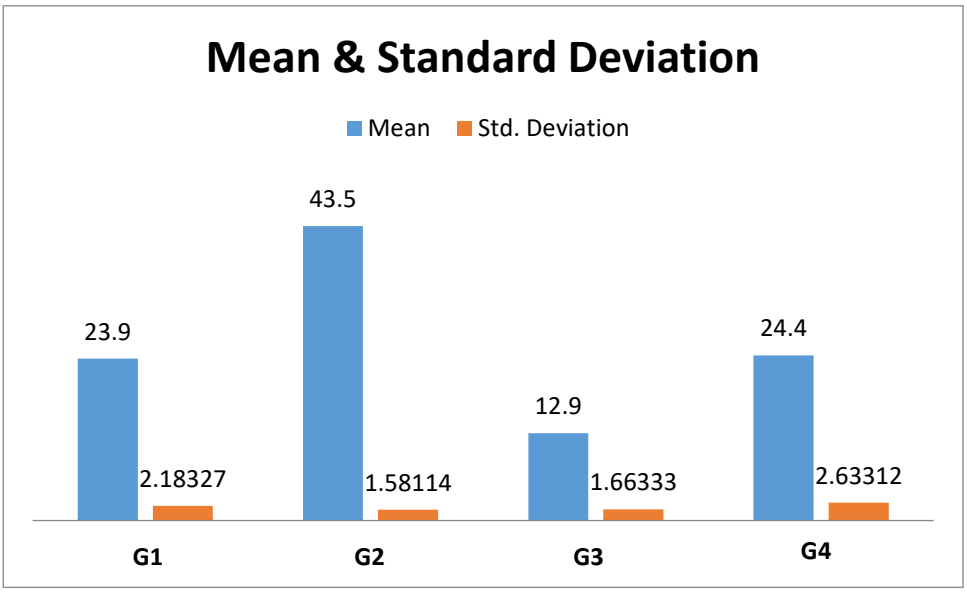
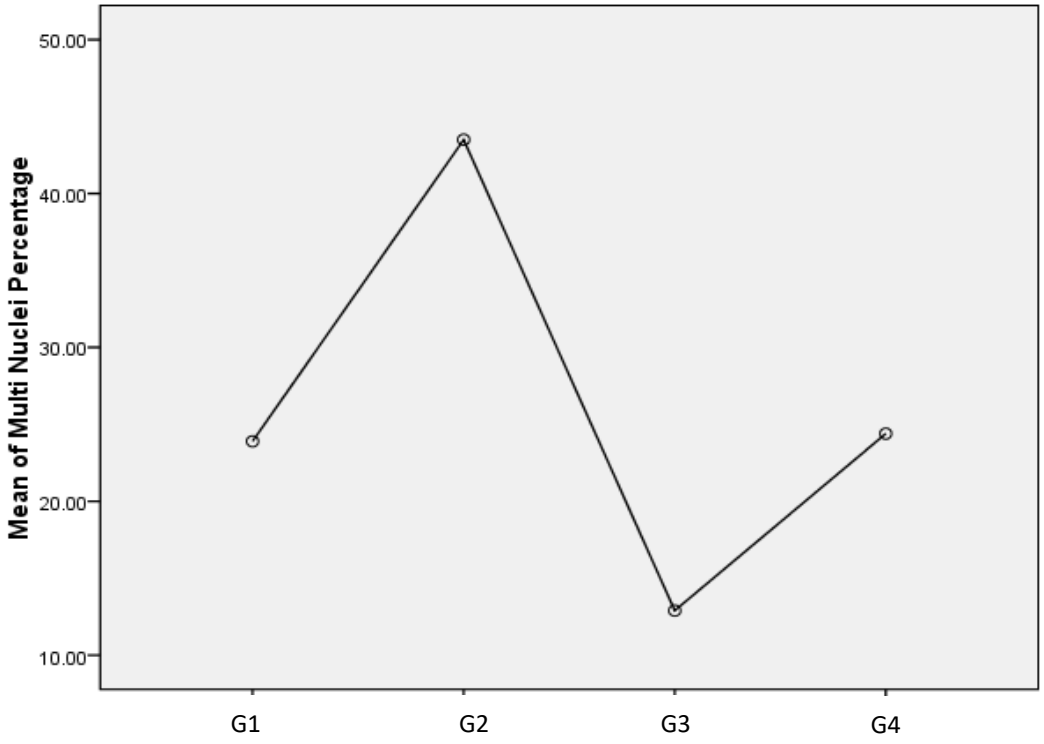


Figure 12: Diagramatic Representation of Multinuclei Percentage



Graph 3: Mean and Standard deviation (Total Micronuclei Percentage)

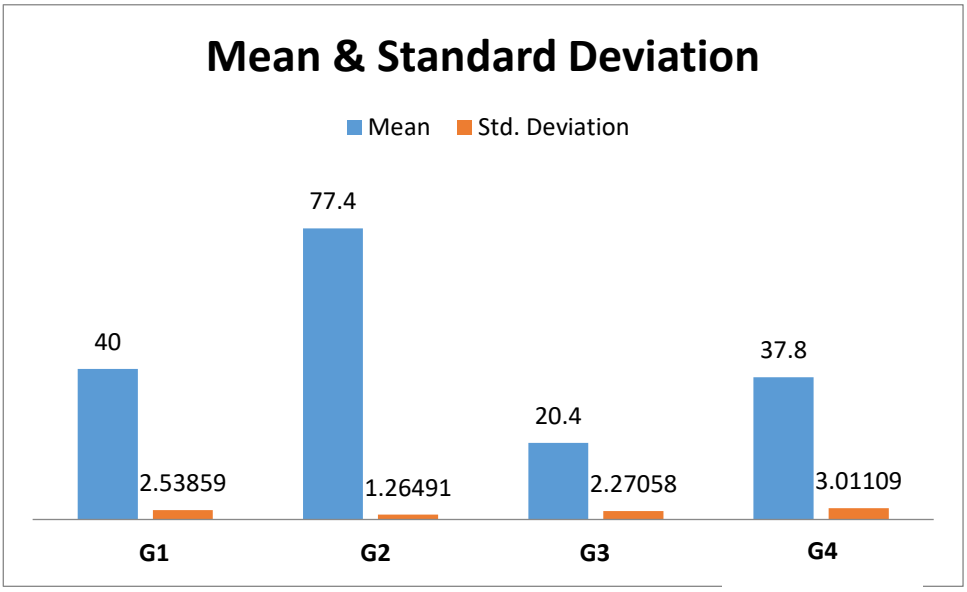
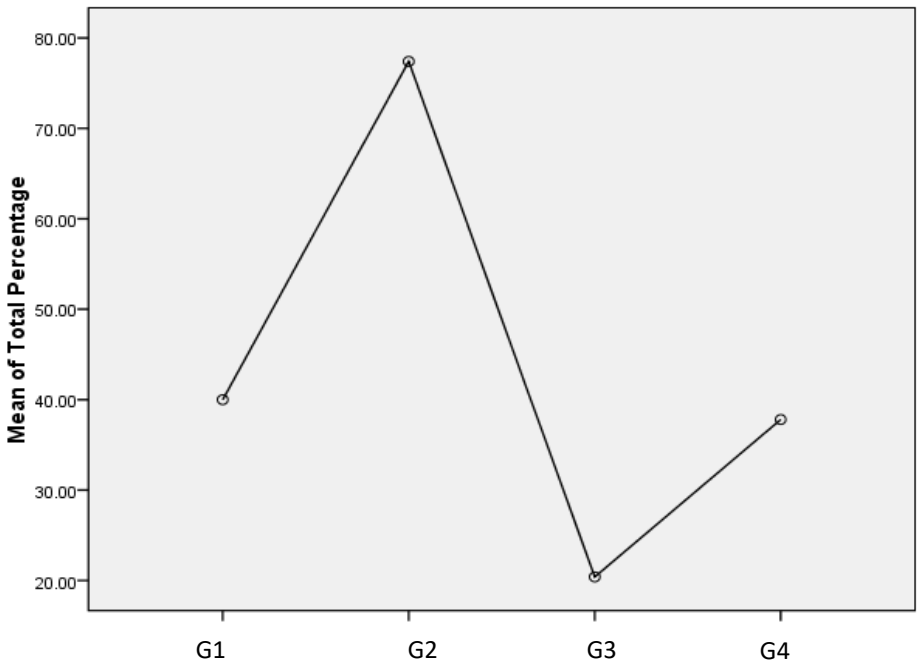


Figure 13: Diagramatic Representation of Total Micronuclei Percentage



ONE WAY ANOVA

One way ANOVA showed significant value 0.000 (p=0.000) which is below 0.05 (ie α = 0.05). Therefore, there is statistically significant difference between the Groups determined by F and p = 0.00.

Trypan Blue Assay

Table 2: Mean and Standard deviation Of Percentage of Viable Cells

PERCENTAGE OF VIABLE CELLS										
					95% Confidence Interval for Mean					
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	df	Mean Square	F	Sig.
G1	10	52.116	1.24960	0.39516	51.2221	53.0099	4	7963.599	6978.00	0.00
G2	10	22.817	1.00587	0.31808	22.0974	23.5366				
G3	10	73.594	1.62362	0.51343	72.4325	74.7555				
G4	10	52.570	0.69041	0.21833	52.0761	53.0639				
G5	10	98.739	0.14098	0.04458	98.6381	98.8399				
Total	50	59.967	25.5174	3.60871	52.7152	67.2192				

Graph 4: Mean and Standard deviation of Percentage of Viable Cells

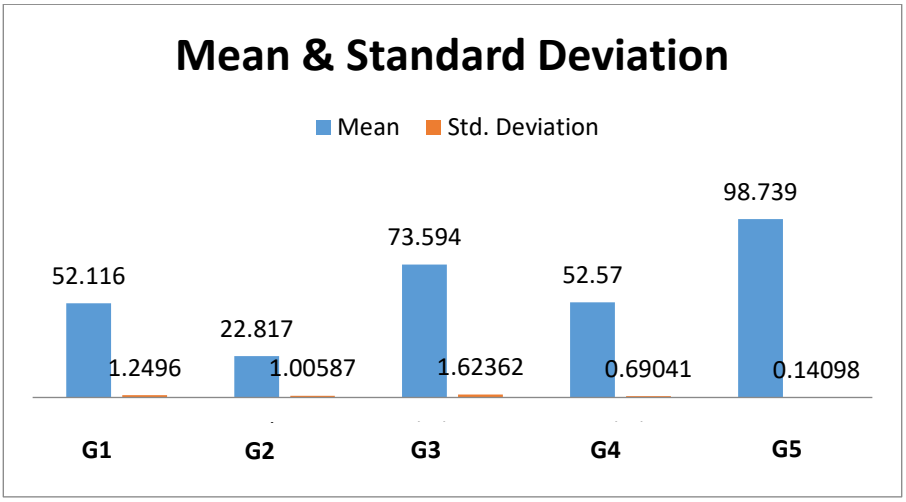
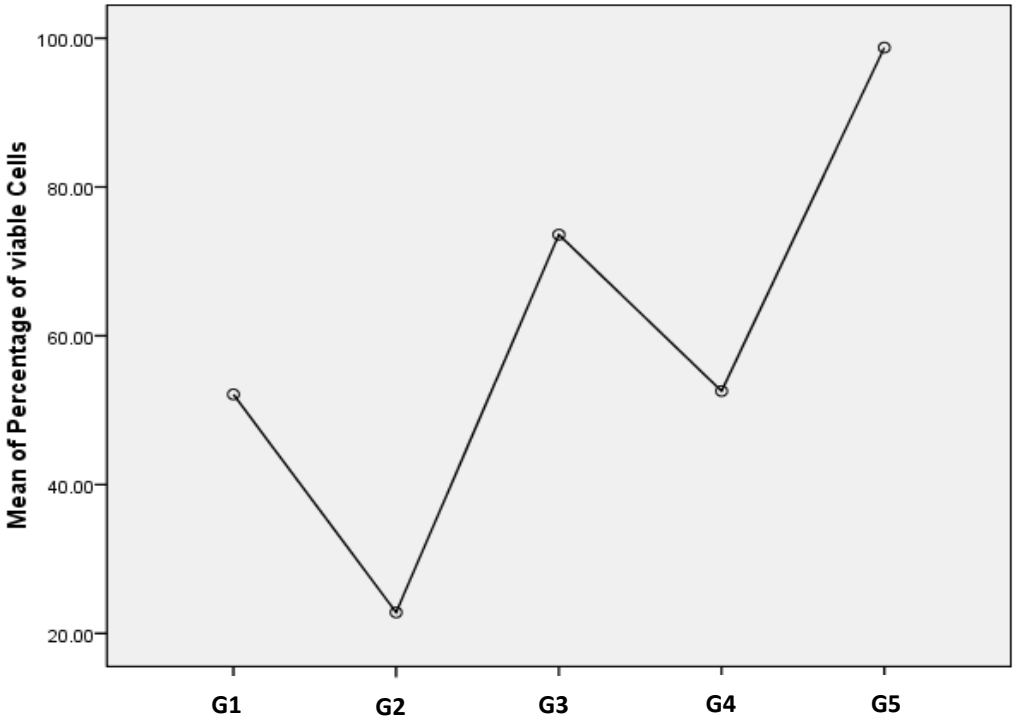


Figure 14: Diagramatic Representation of Percentage of Viable Cells



ONE WAY ANOVA

One way ANOVA showed significant value 0.000 (p=0.000) which is below 0.05 (ie α = 0.05). Therefore, there is a statistically significant difference between Groups determined by F and p = 0.00.

KRUSKAL-WALLIS TEST

The Kruskal Wallis test was done to find out the significant difference between the given groups.

Table 3: Kruskal-Wallis Test

	Ranks		
	Groups	**N**	**Mean Rank**
Percentage of viable Cells	G1	10	19.4
	G2	10	5.5
	G3	10	35.5
	G4	10	21.6
	G5	10	45.5
	Total	50	

Table 4: Test Statistics

	Percentage of Viable Cells
Chi-Square	44.824
df	4
P value	**0.00**
a. Kruskal Wallis Test	
b. Grouping Variable: Group 2	

POST HOC TESTS

The Tukey HSD post hoc analysis was done to find out the significant difference between any two given groups.

Table 5: Binuclei Percentage

BI NUCLEI PERCENTAGE						
Tukey HSD						
					95% Confidence Interval	
(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
G1	G2	-17.80000*	0.7771	0.00	-19.8929	-15.7071
	G3	8.60000*	0.7771	0.00	6.5071	10.6929
	G4	2.70000*	0.7771	0.01	0.6071	4.7929
G2	G1	17.80000*	0.7771	0.00	15.7071	19.8929
	G3	26.40000*	0.7771	0.00	24.3071	28.4929
	G4	20.50000*	0.7771	0.00	18.4071	22.5929
G3	G1	-8.60000*	0.7771	0.00	-10.6929	- 6.5071
	G2	-26.40000*	0.7771	0.00	-28.4929	-24.3071
	G4	-5.90000*	0.7771	0.00	- 7.9929	- 3.8071
G4	G1	-2.70000*	0.7771	0.01	- 4.7929	- 0.6071
	G2	-20.50000*	0.7771	0.00	-22.5929	-18.4071
	G3	5.90000*	0.7771	0.00	3.8071	7.9929

***. The mean difference is significant at the 0.05 level.**

Table 6: Multinuclei Percentage

MULTI NUCLEI PERCENTAGE						
Tukey HSD						
					95% Confidence Interval	
(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
G1	G2	-19.60000*	0.92105	0.00	-22.0806	-17.1194
	G3	11.00000*	0.92105	0.00	8.5194	13.4806
	G4	-0.5	0.92105	0.02	-2.9806	1.9806
G2	G1	19.60000*	0.92105	0.00	17.1194	22.0806
	G3	30.60000*	0.92105	0.00	28.1194	33.0806
	G4	19.10000*	0.92105	0.00	16.6194	21.5806
G3	G1	-11.00000*	0.92105	0.00	-13.4806	- 8.5194
	G2	-30.60000*	0.92105	0.00	-33.0806	-28.1194
	G4	-11.50000*	0.92105	0.00	-13.9806	-9.0194
G4	G1	0.5	0.92105	0.02	-1.9806	2.9806
	G2	-19.10000*	0.92105	0.00	-21.5806	-16.6194
	G3	11.50000*	0.92105	0.00	9.0194	13.9806

***. The mean difference is significant at the 0.05 level.**

Table 7: Total Micronuclei Percentage

TOTAL MICRONUCLEI PERCENTAGE						
Tukey HSD						
					95% Confidence Interval	
(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
G1	G2	-37.40000*	1.05515	0.00	-40.2417	-34.5583
	G3	19.60000*	1.05515	0.00	16.7583	22.4417
	G4	2.2	1.05515	0.01	-0.6417	5.0417
G2	G1	37.40000*	1.05515	0.00	34.5583	40.2417
	G3	57.00000*	1.05515	0.00	54.1583	59.8417
	G4	39.60000*	1.05515	0.00	36.7583	42.4417
G3	G1	-19.60000*	1.05515	0.00	-22.4417	-16.7583
	G2	-57.00000*	1.05515	0.00	-59.8417	-54.1583
	G4	-17.40000*	1.05515	0.00	-20.2417	-14.5583
G4	G1	-2.2	1.05515	0.01	-5.0417	0.6417
	G2	-39.60000*	1.05515	0.00	-42.4417	-36.7583
	G3	17.40000*	1.05515	0.00	14.5583	20.2417

*. The mean difference is significant at the 0.05 level.

Table 8: Percentage of Viable Cells

PERCENTAGE OF VIABLE CELLS						
Tukey HSD						
					95% Confidence Interval	
(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
G1	G2	29.29900*	0.47774	0.00	27.9415	30.6565
	G3	-21.47800*	0.47774	0.00	-22.8355	-20.1205
	G4	-0.454	0.47774	0.01	-1.8115	0.9035
	G5	-46.62300*	0.47774	0.00	-47.9805	-45.2655
G2	G1	-29.29900*	0.47774	0.00	-30.6565	-27.9415
	G3	-50.77700*	0.47774	0.00	-52.1345	-49.4195
	G4	-29.75300*	0.47774	0.00	-31.1105	-28.3955
	G5	-75.92200*	0.47774	0.00	-77.2795	-74.5645
G3	G1	21.47800*	0.47774	0.00	20.1205	22.8355
	G2	50.77700*	0.47774	0.00	49.4195	52.1345
	G4	21.02400*	0.47774	0.00	19.6665	22.3815
	G5	-25.14500*	0.47774	0.00	-26.5025	-23.7875
G4	G1	0.454	0.47774	0.01	-0.9035	1.8115
	G2	29.75300*	0.47774	0.00	28.3955	31.1105
	G3	-21.02400*	0.47774	0.00	-22.3815	-19.6665
	G5	-46.16900*	0.47774	0.00	-47.5265	-44.8115
G5	G1	46.62300*	0.47774	0.00	45.2655	47.9805
	G2	75.92200*	0.47774	0.00	74.5645	77.2795
	G3	25.14500*	0.47774	0.00	23.7875	26.5025
	G4	46.16900*	0.47774	0.00	44.8115	47.5265

*. The mean difference is significant at the 0.05 level.

RESULTS

RESULTS

In the CBMN assay for evaluating genotoxicity, the highest Binuclei percentage mean value was recorded for Group 2 (33.9±1.91195) while Group 3 showed the lowest Binuclei percentage (7.5±1.17851).

The highest Multinuclei percentage mean value was recorded for Group 2 (43.5 ± 1.58114) while Group 3 showed the lowest Multinuclei percentage (12.9±1.66333).

The highest total Micronuclei percentage (derived by the summation of Binuclei percentages and Multinuclei percentages) mean value was recorded for Group 2 (77.4 ± 1.26491) while Group 3 showed the lowest total Micronuclei percentage (20.4±2.27058).

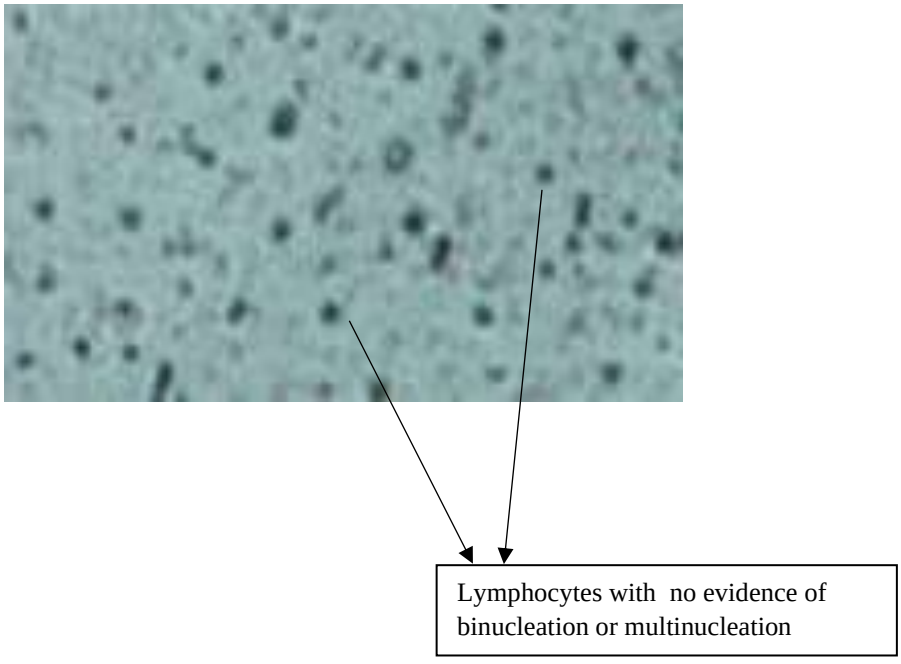


Figure 15: Control

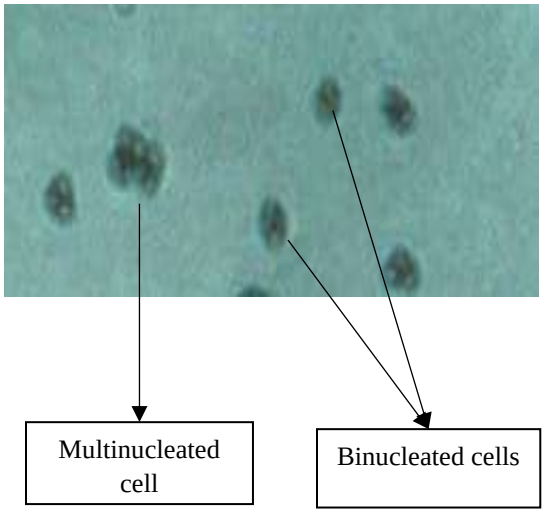


Figure 16: Sodium hypochlorite 2.5%

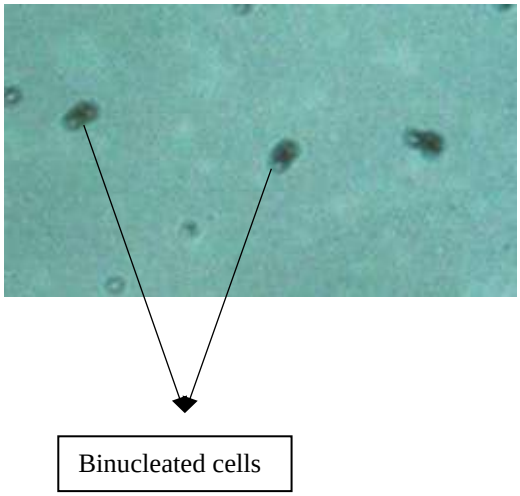


Figure 17: Calcium hypochlorite 2.5%

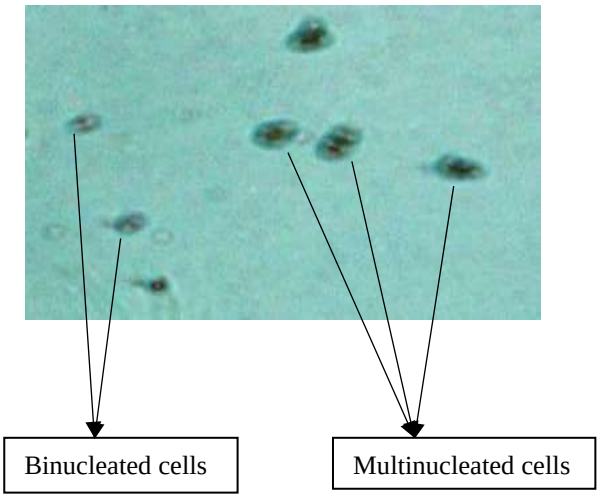


Figure 18: Sodium hypochlorite 5.25%

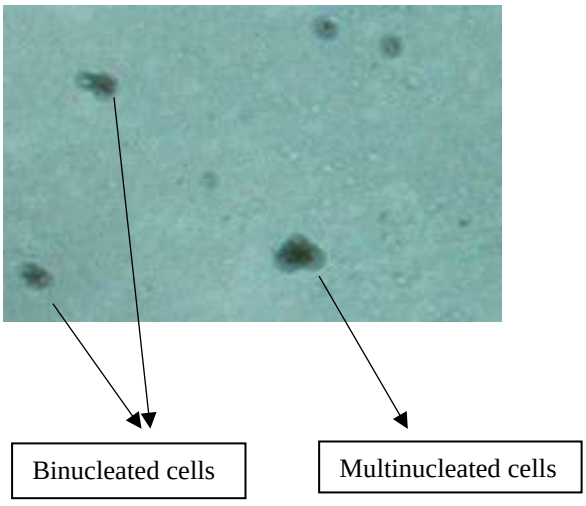


Figure 19: Calcium hypochlorite 5.25%

The trypan blue assay is used for evaluating cytotoxicity, along with cell viability, which is indicated by dye exclusion. The highest percentage of viable cells mean value was recorded for Group 5 (98.739±0.14098). In the test groups, Group 3 showed the highest percentage of viable cells (73.594±1.62362), while the lowest percentage of viable cells was for Group 2 (22.817±1.00587) indicating maximum cytotoxic effects.

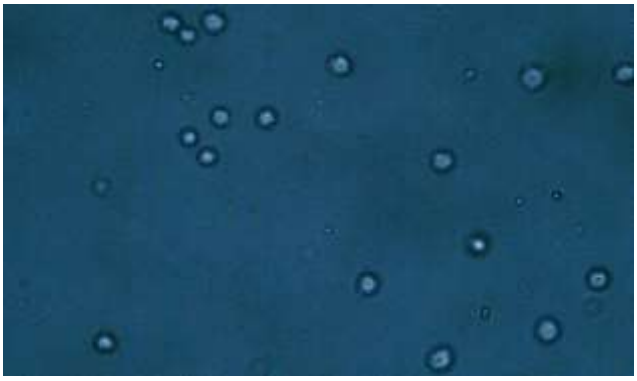


Figure 20: Control

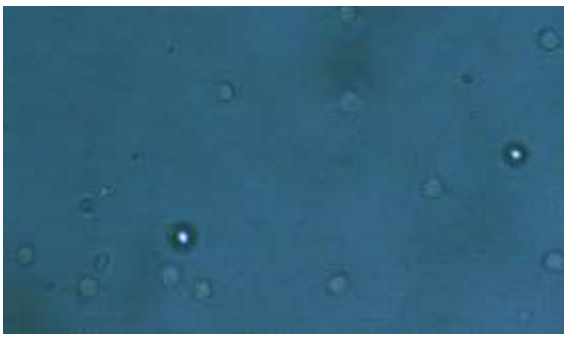


Figure 21: Sodium hypochlorite 2.5%

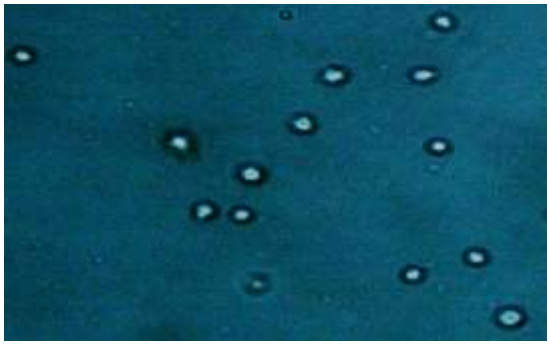


Figure 22: Calcium hypochlorite 2.5%

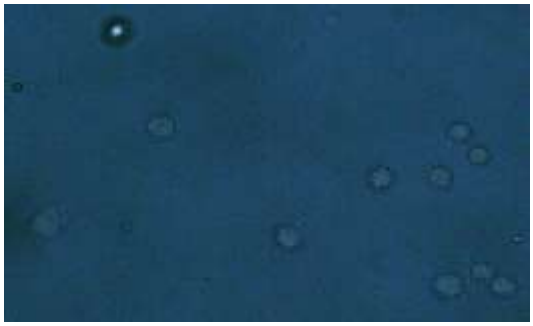


Figure 23: Sodium hypochlorite 5.25%

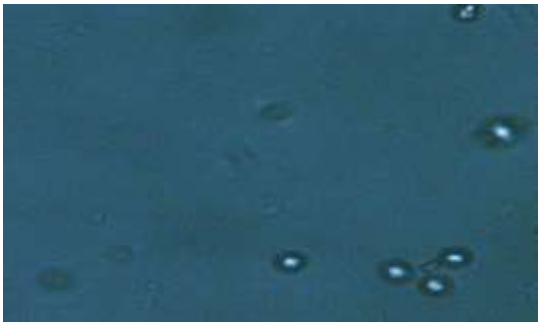


Figure 24: Calcium hypochlorite 5.25%

The Mean and Standard Deviation value for measuring percentage of Genotoxicity (Percentage of Binuclei, Multinuclei and total Micronuclei) of all Groups was tabulated in Table 1. This was then represented separately in Graphs 1 (Binuclei Percentage), Graph 2 (Multinuclei Percentage) and Graph 3 (Total Micronuclei Percentage) and correspondingly shown as Diagrammatic Representations seen in Figures 11, 12 and 13 respectively.

In short, Group 2 showed the highest Binuclei percentage (33%), while Group 3 showed the lowest (7%). Group 2 showed the highest Multinuclei percentage (43%), while Group 3 showed the lowest (12%). Group 2 showed the highest Total Micronuclei percentage (77%), while Group 3 showed the lowest (20%).

This is further evident when we study Figures 16, 17, 18 and 19 with 15 being the Control Group.

The Mean and Standard Deviation value for measuring percentage of Cytotoxicity (Percentage of Viable Cells) of all Groups was tabulated in Table 2. Group 5 which was the Control Group showed 98% of viable cells followed by Group 3 with 72% viable cells and Group 2 with 22% , showing the least percentage of viable cells. This was represented in Graph 4 and was correspondingly shown as Diagrammatic Representation in Figure 14.

Kruskal-Wallis Test for percentage of viable cells (Table 3) showed that besides the Control Group , Group 3 gave the highest value of viable cells indicating that Group 3 showed the least Cytotoxicity.

This is further evident when we study Figures 21, 22, 23 and 24 with 20 being the Control Group

Table 4 shows the Test Statistics with CHI Square of 44.824 , df of 4 and P value of 0.00

One-way ANOVA showed significant difference between the Groups regarding the Binuclei, Multinuclei, Total Micronuclei Percentage, and the percentage of viable cells ($P \leq .05$).

The Tukey HSD post hoc analysis was done to find out the significant difference between the given Groups (Table 5-8).

On comparison of the p value given in the Tables 5 to 8 with ($\alpha = 0.05$), if $p < \alpha$, there is statistical difference between the Groups.

- There is statistically significant difference between Binuclei percentages of Group 1 and Group 4 since p= 0.01 which is less than 0.05 i.e. α.
- There is statistically significant difference of Multinuclei percentages between Group 1 and Group 4 since p= 0.02 which is less than 0.05 i.e. α.
- There is statistically significant difference of Total Micronuclei percentages between Group 1and Group 4 since p= 0.01 which is less than 0.05 i.e. α.
- There is statistically significant difference of Percentage of Viable cells between Group 1 and Group 4 since p= 0.01 which is less than 0.05 i.e. α.

DISCUSSION

DISCUSSION

One of the greatest challenges in endodontic therapy is the procedure of rendering a complex root canal system and its ramifications completely clean of organic and inorganic debris, thereby creating a healthy environment for the tooth to achieve maximal healing. The elimination of microorganisms from the root canal is an important step in the success of endodontic therapy.[62] The colonisation of dentinal walls with biofilm, along with the anatomical complexity of the root canal and the possibility of invasion of dentinal tubules, can compromise the success of endodontic therapy.[63]

Chemo-mechanical preparation plays an important role in the success of endodontic treatment.[64] An ideal endodontic irrigant must present some important characteristics, including antimicrobial activity and tissue-dissolving capacity. Irrigation solutions can cause complications, like tissue damage, allergies, and variable degrees of discomfort to patients which depends on the type and volume of the irrigant used on periradicular tissues.[65] Additionally, it should induce mild or no inflammatory response in the periapical tissues with a minimal toxic effect.[66] Therefore, biocompatibility issue is as important as the antibacterial or tissue-dissolving property for an intracanal irrigation solution.[67]

Since 1920, NaOCl is one of the most used endodontic irrigants. It is known for its antibacterial activity and for its capacity of dissolving organic tissue in root canal.[68] Sodium hypochlorite exhibits a dynamic balance as shown by the following reaction:[69]

$$NaOCl + H_2O \longrightarrow NaOH + HOCl \longrightarrow Na^+ + OH^- + H^+ + OCl^-$$

Interpreting these chemical reactions, sodium hypochlorite acts as a solvent for organic and fat degrading fatty acids, transforming them into fatty acid salts (soap) and glycerol (alcohol) that reduces the surface tension of the remaining solution. [69] NaOCl ionizes to liberate hypochlorous acid (HOCl) and hydroxyl ions in an aqueous environment. HOCl disrupts the microbial metabolism by oxidation of sulfhydryl groups within

bacterial enzyme systems.[70] Saponification, amino acid neutralization, and chloramination reactions contribute to tissue dissolution with participation from hydroxyl ions in the first two reactions and HOCl in the third. The state of HOCl is dependent on the pH of the solution. At pH >8.5, hypochlorite ions (OCl^-) predominate, whereas at pH <6.5 the HOCl molecule is dominant. At pH values between 6.5 and 8.5, they are in a state of equilibrium. HOCl and OCl^- contribute to the available chlorine content of the solution although the HOCl molecule is more active. When hydroxyl ion levels decrease as a result of the saponification and amino acid neutralization reactions, the pH also decreases, thereby favouring the formation of HOCl molecules. The chloramination reaction is then initiated, which is the most important step for tissue dissolution because it results in degradation and hydrolysis of amino acids. The amino acid chloramination reaction forming chloramines interfere with cellular metabolism. Oxidation promotes irreversible bacterial enzymatic inhibition replacing hydrogen with chlorine. This enzyme inactivation can be observed in the reaction of chlorine with amino groups (NH2-) and an irreversible oxidation of sulphydryl groups (SH) of bacterial enzymes (cystein).[71] Strong basic pH and high percentage of free chlorine in solution are the two peculiar actions related to the antibacterial and solvent actions of NaOCl.[71] It has limited activity on the inorganic components of the smear layer, and this required the use of chelating agents.[72] The high pH of sodium hypochlorite interferes in the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation observed in lipidic peroxidation.[70]

Although higher concentrations of NaOCl significantly improve its antimicrobial and tissue-dissolving effects, it can become more cytotoxic and induce inflammatory response when in contact with periapical tissues.[73] It also has a pronounced negative effect on the survival and differentiation of stem cells of the apical papilla, factors which may hinder periapical repair and pulpal regeneration.[74]

Most complications of sodium hypochlorite appear to be the results of its accidental injection beyond the root apex which may cause violent tissue reactions characterised by pain, swelling, haemorrhage, and in some cases the development of secondary

infection and paraesthesia.[75] A great deal of care should therefore be exercised when using sodium hypochlorite during endodontic irrigation. Ehrich *et al*.[76] suggested that a clinician should check, both clinically and radiographically for immature apices, root resorption, apical perforations or any other conditions that may result in larger than normal volumes of irrigant being extruded from the root-canal system into the surrounding tissue. Irrigation should be performed slowly with gentle movement of the needle to ensure that it is not binding in the canal.[77] In an *in vitro* study by Brown *et al*.[78], the use of a reservoir of irrigation fluid in the coronal access cavity and carried into the root canal during filing resulted in significantly less apical extrusion of irrigation solution than with deep delivery with an irrigation needle.

Another important limitation to the use of NaOCl is its chemical instability. External agents like temperature, light, and storage conditions influence the availability of chlorine ions, which successively affect the maintenance and preservation of its properties and influence the outcome of endodontic treatment.[79] Currently, there is no root canal irrigant considered ideal, and alternative solutions continue to be studied.

Calcium hypochlorite ($Ca(OCl)_2$) is a halogenated compound, used for industry sterilization, bleaching, and water purification. In contrast with NaOCl, $Ca(OCl)_2$ is relatively stable with an available chlorine ion percentage higher than NaOCl(up to 65% available chlorine).[80] $Ca(OCl)_2$ has the ability to promote soft-tissue dissolution and presents similar antibacterial properties when compared with NaOCl on Enterococcus faecalis colony-forming units in infected bovine teeth.[74]

$Ca(OCl)_2$ in a freshly prepared aqueous solution, the following reaction occurs:

$$Ca(OCl)_2 + 2\ H_2O \longrightarrow 2\ HOCl + Ca(OH)_2$$

The presence of the Ca^{2+} leads to the production of twice as many hydroxyl ions than in a NaOCl solution. In a $Ca(OCl)_2$ solution, these would take longer to be exhausted in the saponification and amino acid neutralization reactions before the pH could

decrease and favour the formation of hypochlorous acid, which is the more reactive species formed during the ionization reaction.

The preparation of a $Ca(OCl)_2$ solution could also be more accurate than that of NaOCl, because $Ca(OCl)_2$ powder are often weighed and incorporated into water prior to use. On the other hand, a NaOCl solution is prepared by diluting a more concentrated and therefore unstable solution, thus making it difficult to obtain an accurate concentration of NaOCl.[81]

The aim of this study was to assess the cytotoxicity and genotoxicity of NaOCl and $Ca(OCl)_2$ on human peripheral blood cells. This kind of study is important because the root canal irrigant may reach the periapical tissues[16] and influence the prognosis of endodontic therapy, mainly in teeth with destroyed apical constriction because of root canal instrumentation or root resorption. This becomes more critical when regenerative endodontic protocols are used in immature teeth, since root canal irrigant contacts the periapical tissues, which are essential for regeneration.[82] The null hypothesis was rejected because there have been differences between solutions.

In vitro studies are simple, inexpensive to perform, provide a significant amount of data, are often conducted under controlled conditions and can elucidate the mechanisms of cellular toxicity. Cell culture studies are commonly used in the evaluation of genotoxicity and cytotoxicity. The results obtained from these *in vitro* assays might be indicative of the effects observed *in vivo*.[83]

Blattes et al.[47] compared the cytotoxicity of $Ca(OCl)_2$ and NaOCl both in vitro over 3T3 fibroblasts and in vivo through the inflammatory response in both n rats. $Ca(OCl)_2$ showed favourable results for in vitro cell viability and induced a minimal inflammatory response. Swelling was observed immediately after injections of 2.5% NaOCl in some sites, which occurred transiently. $Ca(OCl)_2$ induced only a low-level inflammatory response.

The trypan blue is taken into account as an appropriate assay to evaluate cytotoxicity of dental materials. Trypan blue is a dye that is readily absorbed by dead cells owing to the loss of plasmatic cell membrane selectivity. In contrast, live cells remain unstained.[84] Certainly, such data will contribute for a better understanding of the behaviour of these compounds on the cellular system. Many authors assert that the NaOCl cytotoxicity is directly proportional to its concentrations.[85] With trypan blue staining, this outcome was confirmed for NaOCl and $Ca(OCl)_2$. An increase in cytotoxicity was observed as the concentration increased.[86] In the present study, the solution of 2.5% $Ca(OCl)_2$ seemed to show a better cell viability than NaOCl at the same concentration. $Ca(OCl)_2$ produced some evidence of cytotoxicity only at the highest tested concentrations and the same was observed by others.[87] In contrast, NaOCl was severely cytotoxic at practically all tested concentrations in this study, confirming a recent report conducted by Missotten et al.,[88] in which no surviving ocular cells were observed after treatment of 3 min with 0.5% NaOCl *in vitro*. These findings confirmed that the cell membrane was the main target for the toxic agent and that the damage occurred rapidly. Cell number was significantly reduced, and a considerable number of cells appear darker, indicating enhanced cell death on increasing concentrations of both the solutions. Cell viability was significantly reduced by the application of calcium hypochlorite and sodium hypochlorite in a dose dependent manner, indicating cytotoxicity of these chemicals. The maximum reduction in viability was observed with Sodium Hypochlorite 5.25 % followed by Sodium Hypochlorite 2.5 %. The lowest cytotoxicity was observed for Calcium Hypochlorite 2.5 %. Refer Table 2 and 3, Graph 4 and Figures 14, 21 to 24.

DNA damage is an important step in events ranging from carcinogen exposure to cancer, the results represent a potential alert for a correct evaluation of the potential health risks associated with exposure to the compounds that are present in materials used in clinical practice. Micronuclei (MN) are small additional nuclei within human cells that contain chromosome fragments or whole chromosomes that are excluded from the main nuclei during nuclear division because they could not engage the spindle and segregate properly to the daughter nuclei during the metaphase–anaphase–telophase transition in mitosis. [60] There were other phenomena occurring in the cells,

some of which are difficult to distinguish from classical micronuclei. The following nuclear anomalies indicate genotoxicity: (1) micronuclei (2) broken nuclei, (3) pyknosis, or shrunken nuclei, (4) condensed chromatin, in which the nuclear chromatin appears aggregated, (5) karyorrhexis, or nuclear disintegration involving loss of integrity of the nucleus, and (6) karyolysis, or nuclear dissolution, in which, ghost-like image of the nucleus remains. [89] Micronuclei can be either binucleated or multi-nucleated. The cytokinesis-block micronucleus (CBMN) assay, which measures MN exclusively in cells that have completed one nuclear division ex vivo or in vitro in cultured lymphocytes, is one among the best validated methods for measuring DNA damage in humans. In addition, the number of mono-, bi- and multinucleated cells provides a measure of the cell division rate.[60] In the present study, NaOCl significantly increased the frequency of micronuclei in a dose dependent manner. The results showed that there was a significant correlation between NaOCl concentration and micronuclei frequency (Table 1). The results also showed that there was a significant correlation between $Ca(OCl)_2$ concentration and total micronuclei frequency (Table 1). The highest Binuclei percentage mean value was recorded for Group 2 while Group 3 showed the lowest Binuclei percentage and the highest Multinuclei percentage mean value was recorded for Group 2 while Group 3 showed the lowest Multinuclei percentage. Thus, the highest total Micronuclei percentage was recorded for Group 2 while Group 3 showed the lowest total Micronuclei percentage, suggesting that the most genotoxic of all Groups was Group 2 and the least was Group 3. Refer Graphs 1, 2, 3 and Figures 11, 12, 13, 16 to 19.

From this study, we can suggest that Calcium hypochlorite can be considered as an alternative endodontic irrigant. However, It is important to note that cell culture models have limitations because of the non-physiological conditions in which cells are maintained: only one cell type without cell-cell interaction, no elimination of toxic substances, lack of biotransformation capacity and defense mechanisms.[90] For these reasons, a direct extrapolation of results from cytotoxicity tests to the periapical tissue is not possible.[91] Further *in vivo* researches evaluating the biocompatibility of Calcium hypochlorite solutions is necessary to verify its use in endodontic therapy.

CONCLUSION

CONCLUSION

From the study the following conclusions can be drawn :

1. 5.25% $Ca(OCl)_2$ was found to be less cytotoxic and genotoxic than 5.25% NaOCl.
2. 2.5% $Ca(OCl)_2$ was found to be less cytotoxic and genotoxic than 2.5% NaOCl.
3. 5.25% $Ca(OCl)_2$ was found to be more cytotoxic and genotoxic than 2.5% $Ca(OCl)_2$.
4. 5.25% NaOCl was found to be more cytotoxic and genotoxic than 2.5% NaOCl.

Therefore, based on the observations in this study, we see that Calcium hypochlorite is a relatively safer root canal irrigant than Sodium hypochlorite specifically in terms of cytotoxicity and genotoxicity and can be considered as a possible alternative to Sodium hypochlorite.

SUMMARY

SUMMARY

As the root canal system is complex and unpredictable, using root canal irrigants and medicaments are essential in order to enhance the disinfection of the canal. Sodium hypochlorite is the most common irrigant used in endodontics. Despite its excellent antimicrobial activity and tissue solubility, sodium hypochlorite has tissue toxicity and is chemically unstable. External agents, such as temperature, light and storage conditions can influence the availability of chlorine ions and interfere with its effectiveness. Hence, auxiliary agents should be investigated.

In terms of antimicrobial effect and tissue dissolution, Calcium hypochlorite showed comparable results to that of Sodium hypochlorite. In terms of cytotoxicity and genotoxicity, Calcium hypochlorite showed lesser toxicity levels than Sodium hypochlorite. Thus, based on this study, we can safely assume that Calcium hypochlorite has the potential to be used as a root canal irrigant for endodontic procedures and can be considered as an alternative to Sodium hypochlorite.

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BIBLIOGRAPHY

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ANNEXURE

ST. GREGORIOS DENTAL COLLEGE

UNDER THE MANAGEMENT OF MJSCE TRUST, PUTHENCRUZ

CHELAD , KOTHAMANGALAM, ERNAKULAM DIST, KERALA - 686681

SGDC/152/2019/3732

15/11/2019

ETHICAL CLEARANCE CERTIFICATE

To,

Dr.Pooja Jayan
St.Gregorios Dental College
Chelad,Kothamangalam

Dear Dr.Pooja Jayan,

Subject: Ethics Committee Clearance - reg.

Protocol: Evaluation and comparison of cytotoxicity and genotoxicity of different concentrations of sodium hypochlorite and calcium hypochlorite: an in-vitro study.

After the Institutional Ethics Committee (IEC) held on 15th of November 2019, this study was examined and discussed. After the consideration, the committee had decided to approve and grant clearance for the aforementioned study.

The members who attended the meeting at which the protocol was discussed were:

1) Dr.C.K.K Nair - Former BARC Scientist.
2) Dr.Cinu Thomas A - Scientist, Senior lecturer, Department of Pharmaceutical Sciences Centre for Professional and Advanced Studies.
3) Dr.Lissy Jose - Former member Women's Welfare Association.
4) Adv.Jose Aranjani - Advocate.
5) Dr.Sauganth Paul - Reader, Department of Biochemistry, St.Gregorios Dental College.
6) Dr.Eapen Cherian - Secretary.
7) Dr.Jain Mathew - Principal and Head of the Department, Department of Conservative Dentistry and Endodontics.
8) Dr.George Francis - Head of the Department, Department of Prosthodontics and Crown & Bridge.
9) Dr.Binoy Kurian - Head of the Department, Department of Orthodontics &Dentofacial Orthopaedics.

St.Gregorios Dental College Chelad - 686 681

Dr. C.K.K Nair
Chairman Institutional Ethics Committee
St.Gregorios Dental College, Chelad

Dr.Eapen Cherian
Secretary

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LIST OF ABBREVIATIONS USED

(*In alphabetical order*)

	ABBREVIATIONS	DESCRIPTIONS
1.	ANOVA	Analysis of Variance
2.	CBMN	Cytokinesis Block Micronucleus
3.	CFU	Colony Forming Units
4.	CHX	Chlorhexidine gluconate
5.	$Ca(OCl)_2$	Calcium Hypochlorite
6.	EDTA	Ethylene Diamine Tetra-acetic Acid
7.	FBS	Foetal Bovine Serum
8.	NaOCl	Sodium Hypochlorite
9.	OCT	Octenidine Hydrochloride
10.	OECD	Organisation for Economic Co-operation and Development
11.	PBS	Phosphate Buffered Saline
11.	PDT	Photodynamic Therapy
12.	PUI	Passive Ultrasonic Irrigation
13.	TC	Tissue Culture

BINUCELI PERCENTAGE

Sample No	Groups	Binuclei (%) [Based on 100 cells counted]
1	G1	19
	G2	34
	G3	7
	G4	12
	G5	-
2	G1	17
	G2	32
	G3	9
	G4	14
	G5	-
3	G1	20
	G2	31
	G3	6
	G4	13
	G5	-
4	G1	18
	G2	35
	G3	7
	G4	13
	G5	-
5	G1	16
	G2	33
	G3	9
	G4	11
	G5	-
6	G1	15
	G2	32
	G3	8
	G4	13
	G5	-
7	G1	14
	G2	34
	G3	9
	G4	14

	G5	-
8	G1	13
	G2	35
	G3	7
	G4	15
	G5	-
9	G1	15
	G2	36
	G3	6
	G4	14
	G5	-
10	G1	14
	G2	37
	G3	7
	G4	15
	G5	-

MULTINUCLEI PERCENTAGE

Sample No.	Groups	Multinuclei (%) [Based on 100 cells counted]
1	G1	22
	G2	45
	G3	13
	G4	20
	G5	-
2	G1	23
	G2	46
	G3	15
	G4	25
	G5	-
3	G1	25
	G2	44
	G3	14
	G4	23
	G5	-
4	G1	21

	G2	43
	G3	12
	G4	22
	G5	-
5	G1	25
	G2	44
	G3	16
	G4	28
	G5	-
6	G1	21
	G2	45
	G3	11
	G4	26
	G5	-
7	G1	23
	G2	42
	G3	11
	G4	27
	G5	-
8	G1	26
	G2	43
	G3	12
	G4	24
	G5	-
9	G1	27
	G2	41
	G3	12
	G4	22
	G5	-
10	G1	26
	G2	42
	G3	13
	G4	27
	G5	-

TOTAL MICRONUCLEI PERCENTAGE

Sample no.	Groups	Total (%) [Based on 100 cells counted]
1	G1	41
	G2	79
	G3	20
	G4	32
	G5	-
2	G1	40
	G2	78
	G3	24
	G4	39
	G5	-
3	G1	45
	G2	75
	G3	20
	G4	36
	G5	-
4	G1	39
	G2	78
	G3	19
	G4	35
	G5	-
5	G1	41
	G2	77
	G3	25
	G4	39
	G5	-
6	G1	36
	G2	77
	G3	19
	G4	39
	G5	-
7	G1	37
	G2	76
	G3	20
	G4	41
	G5	-
8	G1	39
	G2	78
	G3	19

	G4	39
	G5	-
9	G1	42
	G2	77
	G3	18
	G4	36
	G5	-
10	G1	40
	G2	79
	G3	20
	G4	42
	G5	-

PERCENTAGE OF VIABLE CELLS

Sample No.	G1	G2	G3	G4	G5
1	52.85	21.97	71.79	52.53	98.67
2	50.57	24.22	75.05	52.02	98.96
3	52.59	21.85	75.82	52.80	98.73
4	51.20	22.54	73.20	51.45	98.54
5	50.95	21.55	71.56	52.75	98.77
6	52.75	22.90	72.80	53.25	98.85
7	53.45	23.45	74.65	52.85	98.58
8	51.80	24.10	75.24	51.74	98.62
9	54.25	23.73	71.55	52.55	98.75
10	50.75	21.86	74.28	53.76	98.92