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RESEARCH ARTICLE

EVALUATION OF TOXICITY OF 0.2% CHLORHEXIDINE GLUCONATE AND 0.15% BENZYDAMINE HCL (COMBINED) WITH 0.2% CHLORHEXIDINE GLUCONATE (ALONE) ON ORAL CELL LINE

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Abstract

Aim: The purpose of this in vitro study wasto evaluate whether the toxic effects are reduced by using the combination of 0.5% Benzydamine hydrochloride along with 0.2% Chlorhexidine gluconate and to compare it with 0.2 % Chlorhexidine gluconate alone.

Materials and Methods: Two groups with a total of 160 cultured cell samples consisting of 80 cultured cell samples each of Control group (0.2% Chlorhexidine Gluconate alone)and Experimental group (Combination of 0.2% Chlorhexidine Gluconate and 0.15% Benzydamine Hydrochloride). Again subdivided as A and Bwith 40 cultured cell samples each, to evaluate viable cell count with dye exclusion test and cell proliferation rate by using MTT assay, for 30 seconds and 60 seconds.

Result: Mean viable cell count andMean proliferation ratewas found to be higher in the experimental groupas compared to the control group when exposed to 30 secondsas compared to 60 seconds (p-value<0.001).

Conclusion: The results of this in vitro study indicated that 0.2% Chlorhexidine Gluconate, when used alone, is more cytotoxic whereas the same concentration of 0.2% Chlorhexidine Gluconate when used in combination with 0.15% Benzydamine Hydrochloride is less toxic to cells thus maintaining their viability.

Clinical Significance: This study adds value to the use of 0.2% chlorhexidine gluconate regimen over the combination therapy to achieve periodontal health.

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Introduction:-

Periodontitis is a destructive inflammatory disease of the supporting tissues of the teeth and is caused either by a specific microorganism or by a group of specific microorganisms. It represents primarily anaerobic Gram-negative oral infection that leads to gingival inflammation, destruction of periodontal tissues, loss of alveolar bone, and eventual exfoliation of teeth in severe cases.[1,2] The pathophysiology behind it is the accumulation of microbial plaque and the host response to it.[3] Plaque is the primary etiologic agent in the development of gingivitis and periodontal diseases.[4]

Chlorhexidine which is routinely used, is a bis bi-guanide antiseptic, containing a variety of active ingredients effective against Gram-positive, Gram-negative bacteria, Viruses, and Yeast.[5] The antimicrobial activity of Chlorhexidine depends on its concentration and the susceptibility of the bacterial species. At low and high concentrations, Chlorhexidine may act as bacteriostatic and bactericidal respectively.[6,7]

Few studies[8,9,10,11]have shown that Chlorhexidine has toxic effects on a variety of eukaryotic cells.Numerous adverse effects mentioned are tooth and restoration staining, soft tissue staining, increased calculus deposition, unpleasant taste, taste alteration, burning sensation, desquamation, and mucosal irritation.A study done by Ebru Olgun Erdemir[12] has mentioned that 0.15% Benzydamine Hydrochloride when used in combination with 0.12% Chlorhexidine helps reduce the cytotoxic effects. In their study,[12]they had used a 0.12% concentration of Chlorhexidine instead of 0.2% Chlorhexidine.The effect of the routinely used concentration (0.2% of Chlorhexidine) in combination with Benzydamine Hydrochloride, however, is not studied yet.

Benzydamine (also known as Tantum Verde and branded in some countries as Difflam), available as the hydrochloride, is a locally-acting non-steroidal anti-inflammatory drug with local anestheticand analgesic properties.[13]It inhibits the oxidative burst and release of granules from neutrophils and thus prevents the lactate dehydrogenase enzyme and maintains the membrane integrity of the cell.[14]

The trypan blue is a routinely used vital stainderived from toluidine that selectively colors the dead tissues or cells, blue.However, this trypan blue does transverse the membrane of the dead cells. Hence, dead cells show a distinct blue color under a microscope. Since live cells are excluded from staining, this staining method is also described as a Dye Exclusion Method.In this method, cell viability is determined by counting the unstained cells under a microscope.

This, in vitro study, was therefore planned to evaluate and compare the toxic effects of the combination of 0.2% Chlorhexidine Gluconate and 0.15% Benzydamine Hydrochloride with 0.2% Chlorhexidine Gluconate alone on the oral cell line.

Materials And Methods:-

The study was carried out after getting approval from the institutional ethical committee at Department of Periodontology, Bharati Vidyapeeth Deemed to be University, Dental College and Hospital, Pune in August 2019. The Smulow-Glickman (S-G) gingival epithelialcell line was obtained from NCCS (National Center for Cell Science) Pune. The cell line was maintained under standard conditions. Cells were cultured using Dulbecco's Modified Eagle Media (DMEM) supplemented with Penicillin G (100units/ml) and streptomycin(100µg/ml), and 10% Fetal Bovine Serum(FBS). Incubation was done at 37ºc in an atmosphere of 5% carbon dioxide or 95% air in 100% humidity in the incubator.Once the cells were confluent, the medium was removed. The cell layer was washed with Phosphate Buffered Saline (1X). 0.25% trypsin-EDTA solution was added and incubated for 3 min in a 5% Carbon dioxide incubator to detach the cells.4ml of 10% Dulbecco's Modified Eagle Media (DMEM) was added to it. This solution was taken in a 15 ml sterile test tube and then centrifuged around 2000rpm for 3 minutes.The supernatant in the test tube was discarded. Again 1ml of DMEM was added by pipetting to the remnant which remained at the base to get a uniform cell suspension which was used for carrying out the assay after cell counting was done. For this, 5 µl of cell suspension was used to which 45 µl of trypan blue dye was added. This 50 µl solution was kept in a centrifugal sterile tube. Out of this 10µl was loaded in Neubauer chamber and then cell counting was done by using a microscope. The total number of cells found in $1mm^2$ was between 20-50 cells. To find out the exact amount of the cell solution to be used for the assay, the formula used was: $n_1.v_1=n_2.v_2$,and calculation was done.

The study consisted of two groups: Control group- (0.2% Chlorhexidine Gluconate alone) and Experimental group (Combination of 0.2% Chlorhexidine Gluconate and 0.15% Benzydamine Hydrochloride) with a total of 160 cultured cell samples, with 80 cultured cell samples each. Samples were again subdivided into 40 cultured cell samples for evaluation of viable cell count by Dye Exclusion Test (using a vital dye "Trypan blue") and B- 40 cultured cell samples for evaluation of cell proliferation rate by using MTT (Methyl-Tetrazolium) Assay (Viable cells reduced the MTT reagent to colored "Formazan" products). Samples of subgroup A and subgroup B were again equally divided into 20 cultured samples, and they were categrised into tests for 30 seconds and 60 seconds each respectively.Cell proliferation rate was determined by MTT assay.

The statistical analysis was carried out by using the Parametric Significance Test (Student't'-test - Paired and Unpaired)using SPSS (Statistical Package for social sciences) Version 25:0.

Results:-

The cell viability and cell proliferation rate were checked for the cytotoxic effect using Chlorhexidine alone and by addition of Benzydamine Hydrochloride.It was found that there was a statistically significant difference between the control and the experimental groups.

Table 1, Graph 1 showsthe Mean Viable cell count between two groups at 30 seconds and 60 seconds. At 30 seconds of exposure in the Control group, it was 1.790 ± 0.2360 and in the Experimental group,it was 2.315 ± 0.2159(p-value of<0.001). At 60 seconds inthe Control group,it was 1.300 ± 0.1026 and in the Experimental group,it was 2.185 ± 0.1927 (p-value of<0.001). It was statistically significant. The Mean viable cell count at 30 seconds is more in the Control group and more or less equal in the Experimental group as compared after 60 seconds of exposure.

Table 2 Graph 2 compares the Mean Cell proliferation rate between the two groups at 30 and 60 seconds of exposure. At 30 seconds of exposure in the Control group, it was 0.01525 ± 0.0009665 and in the Experimental group,it was 0.02635 ± 0.002007 (p-value of<0.0001).After 60 seconds of exposure in the Control group was 0.0149 ± 0.0007182 and in the Experimental group was 0.0263 ± 0.004868 (p-value of<0.0001). The Mean cell proliferative rate at 30 seconds is more in the Control group and more or less equal in the Experimental group as compared after 60 seconds of exposure.

The results of the present study demonstrated that the Experimental group had more cell proliferation rate as compared with the Control group, which is in agreement with the study of Cristina Trigo Cabral &Maria Helena Fernandes 2007[18] where comparison of Chlorhexidine (CHX) and Povidone–iodine on the human alveolar bone cells was done. Chlorhexidine with 0.12 % and0.2% concentrations and the concentration of Povidone-iodine was 5% and 10% which was exposed for 2 minutes.

Discussion:-

Due to its recognized antimicrobial and other beneficial properties, Chlorhexidine, in few studies, has demonstrated its toxic effects on eukaryotic cells. Isis R. Sanchez et al 1988[15]carried out a study to evaluate the cytotoxicity of 2%Chlorhexidine diacetate and10% of Povidone-iodine on canine embryonic fibroblasts. Cell viability was assessed by using trypan blue. It was found that the canine embryonic fibroblast did not survive on exposure to 24 hours to Chlorhexidine with concentrations of 0.013% and greater.They concluded that Chlorhexidine dilution of 0.006% or less was safer for canine fibroblasts.Jeffery J. Pucher and Jon C. Daniel 1993[11]conducted an in vitro study on the effects of chlorhexidine digluconate on human fibroblasts. Cells were exposed for an hour to 0.002% and 0.005% concentrations of Chlorhexidine and they were exposed for 30 seconds to 0.12% concentration of Chlorhexidine. The cell viability was determined by trypan blue staining. On exposure for one hour, 90% of the cells remained viable only with a 0.002% concentration of Chlorhexidine. The 0.005% and 0.12% concentrations of Chlorhexidine showed high cytotoxicity to human fibroblast. ..Ghabanchi J et al 2013[16] conducted a study to determine the cytotoxic effect of three commercial types of mouthwash Chlorhexidine, Persica (Indole, Alkaloids, Flavonoids, Sulphur containing compounds, Tropacolin and Phytosterol) and Irsha (Alcohol, Glycerin, Sodium lauryl sulfate (SLS), Benzoic acid and Allantoin) on the cultured fibroblasts. The extent of cytotoxicity was confirmed by Trypan blue dye exclusion method. The different concentrations (2, 4, 8, 16, 32, 64, 128) of mouthwashes were diluted up to 1:128 for 1, 2, 3, 4 days.Cytotoxicity of all three types of mouthwash was reduced by increasing the dilutions. They concluded that 1:32 dilution of Chlorhexidine was cytotoxic to fibroblast cells.

In the present study, cell viability with the Experimental group was more as compared to the Control group, and also more cell viable count was seen after 30 seconds of exposure.The findings of our study are in agreement with the study by Ebru OlgunErdemir 2007[12]who had checked cytotoxicity by using Micronucleus Test and concentration used was 0.12% Chlorhexidine Gluconate with 0.15% Benzydamine Hydrochloride at 0 days and 7-day exposure.The cell proliferation assays were used to determine the metabolic activity or enzymatic activity present within the cells. It acts as a marker of viable cells. There are different assays used to check the cell proliferation rate.MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue] is a water-soluble tetrazolium salt. The dye is reduced by the mitochondrial enzyme succinate dehydrogenase to produce a coloredformazan product in live cells.Viable cells with active metabolism convert MTT into a purple-colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan. Thuscolor formation serves as a useful and convenient marker of only the viable cells.

Regarding cell proliferation rate, significant alterations were observed. Fernanda Campos Rosetti et al 2010[17]evaluated the cytotoxic effects of Chlorhexidine with 0.06%,0.12%, 0.2%,1% and 2% concentrations on cultured odontoblast-like cells (MDPC-23). The MDPC-23 cells were exposed to contact with the CHX solutions for different times:60 s, 2 h and 60 s with a recovery period of 24 h. Cell metabolism was determined by MTT Assay and found that there was a decrease in cell metabolism by 61%, 63%, 65%, 67%, and 70% respectively. All Chlorhexidine concentrations were more toxic to cultured MDPC-23 cells after a 2-h exposure time compared to exposure of 60 seconds. They concluded that regardless of the concentration, the longer contact time of the cells with Chlorhexidine, the more intense the cytotoxic effects.Thus, they concluded that both concentrations of Chlorhexidine causes cell death after 2 minutes of exposure and have deleterious effects on cell proliferation.

Conclusion:-

From the above observations, it can be concluded that there was a more viable cell count and high proliferation rate when the Experimental solution was used.Thus, it indicates that 0.2% Chlorhexidine Gluconate, when used alone, is more cytotoxic whereas the same concentration of 0.2% Chlorhexidine Gluconatewhen used in combination with 0.15% Benzydamine Hydrochloride is less toxic to cells - maintaining their viability.

More research is however needed to validate the same as there was limited sample size for this study.

Tables:

Table 1:- Comparison of Mean Viability Count between Control Group and Experimental Group

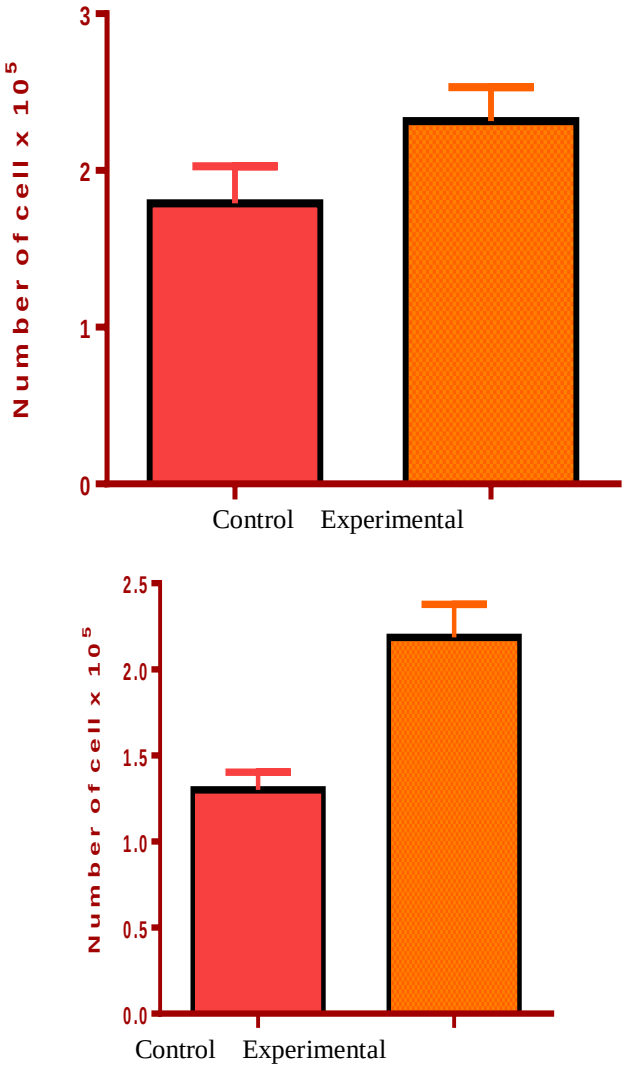
Groups	Time Interval	Mean	Standard Deviation	t-Value	p-Value
Control group	30-Secs	1.79	0.24	8.258	<0.001
	60-Secs	1.30	0.10		
Experimental group	30-Secs	2.32	0.22	1.324	<0.001
	60-Secs	2.19	0.19		

Table 2:- Comparison of Cell Proliferative Rate Between Control Group and Experimental Group.

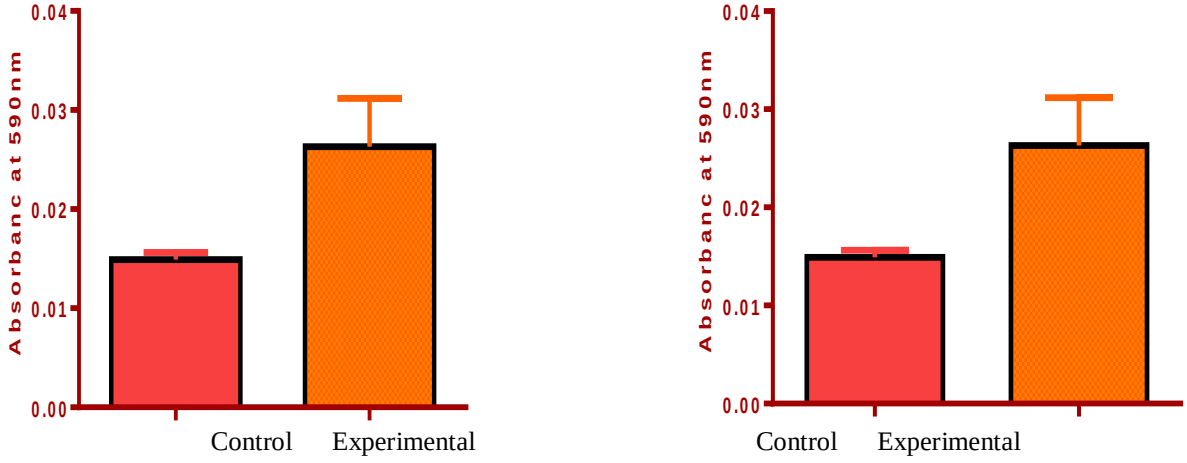
Groups	Time Interval	Mean	Standard Deviation	t-Value	p-Value
Control Group	30-Seconds	0.0153	0.00097	2.392	0.027
	60-Seconds	0.0149	0.00072		
Experimental group	30-Seconds	0.0264	0.0020	0.051	0.960
	60-Seconds	0.0263	0.0049		

Graphs

Graph 1:- Graphical representation of Mean Viable cell count.



Graph 2:- Graphical representation of mean cell proliferation rate.



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Legends

1. TABLE 1: Comparison of Mean Viability Count between Control Group and Experimental Group
2. TABLE 2: Comparison of Cell Proliferative Rate Between Control Group and Experimental Group
3. GRAPH 1: Graphical representation of Mean Viable cell count.
4. GRAPH 2: Graphical representation of mean cell proliferation rate.