Investigation of Cellular and Tissue Cytotoxicity of Modified Chlorehexidine as an Intracanal Irrigant; An In-Vitro and Ex-Vivo Study

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Abstract

Background & Objectives: Sodium hypochlorite is the first choice among the cleaners given its antibacterial properties and tissue solubility. On the other hand, chlorhexidine competes with it in all aspects except for tissue solubility. Surface-active agents or surfactants are compounds used as emulsifiers, irrigants, and accelerators of tissue solubility. Sodium lauryl ether sulfate was the surfactant used in the study and the purpose of the study was to examine the cellular and tissue cytotoxicity of modified chlorhexidine as an intracanal irrigant in vitro and ex-vivo.

Materials and methods: For evaluating the tissue cytotoxicity in this study, 16 male rats were selected, divided into four groups of four, and each day, one of them was implanted under the skin. After anesthesia and shaving the back hair, three separate incisions were made at two cm distance. Then the tested materials (modified chlorhexidine, chlorhexidine, and sodium hypochlorite) were placed inside poly ethylene tubes with an inner diameter of 1.1 mm and a length of one centimeter and a separate polyethylene tube containing the material was inserted in each cut. The animals were then slowly killed by thiopental injection of 0.5% (0.05%) and sampling was carried out to prepare a histological slide. The samples were placed in 10% formalin for two weeks and then the slides were prepared. After slide preparation, only the number of inflammatory cells was counted and scored according to Robbins classification. One must note that, the type of inflammatory cells was not considered in this study and only their accumulation values, 25%, 50%, 75% and more than 75%, were examined. The slide was examined in a Hi Power field. The results were analyzed by non-parametric Kruskal Wallis and Mann Whitney tests.

Results: The toxicity of the solutions tested on fibroblast cells for groups one to four is, respectively, 27.5, 56, 69, 25, 41.75 and from right to left. The results of comparing the means in four groups at level 4 of Landaindicated that the groups do not differ significantly in terms of toxicity. Considering the obtained statistical results, there was a significant difference in terms of histological reaction between the materials used and the histotoxicity of the modified chlorhexidine and chlorhexidine was significantly less than sodium hypochlorite. In terms of time, there was a significant difference between the time periods of 7 and 30 days, 7 and 60 days, 15 and 60 days, and 30 and 60 days.

Conclusion: The results indicated that the resulting compound could be used as a suitable candidate to replace sodium hypochlorite given its disadvantages, which calls for more studies.

Keywords: Sodium Hypochlorite, Chlorhexidine, Modified Chlorhexidine, Cytotoxicity, Tissue Cytotoxicity.

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INTRODUCTION

Non-surgical endodontic therapy is a predictable method for tooth preservation that will be extracted if not treated. The success of endodontic therapy with a vital pulp is more than that of a necrotic tooth with a periradicular lesion. This difference is because of the stimulation of the remaining necrotic tissue and the inability to remove microorganisms and their products. Successful endodontic therapy is based on making a correct diagnosis and an appropriate treatment plan using knowledge of morphology and tooth shape (1). The key elements in endodontic therapy are the anatomy and shape of teeth, tools and equipment, the ability of irrigants and filling the root canal (2,1). Reaching a successful endodontics calls for proper preparation and shaping of the root canal system (11,12). Microscopic studies have indicated that the root canal morphology is complex (1,3,13) and lateral canals, canal curvatures, inaccessible areas, and isthmus make complete canal clearance impossible (1,3,14). Over time, it has been clarified that the primary etiological factor in the formation of pulp and periapical lesions are bacteria (2,4,15,16,17).

Even though about 500 species of bacteria have been identified in the oral environment, only a limited number of them can be colonized in the root canal system (18). When the dental pulp undergoes pathological changes because of trauma or decay, the root canal system becomes susceptible to infection by different species of bacteria via toxins and their products (13).

The microbial flora along the necrotic root canal depends on the stage of infection. At the onset of the infection, there are optional bacteria in the environment that survive in both aerobic and anaerobic conditions. As the infection progresses (over three months), the bacterial flora progresses to anaerobes, which can live in oxygen-free conditions (18, 19). Microbial flora of the primary endodontic infection is often polymicrobial, gram-negative, and anaerobic (20) and is sensitive to the antimicrobial activity of different intracanal drugs (17). Microorganisms could exist in the root canal, dentinal tubules, subcutaneous canals, and apical canal (18: 6-2). In some teeth, even the dentin is infected with DEJ (19). If the cleaning is not done properly, the necrotic residues of the soft tissue act as a food source for the remaining bacteria and can lead to re-contamination of the canal (13,14,21). Thus, the first and most important goal in endodontic therapy is the complete removal of microorganisms from the root canal system and providing an environment for the repair of periapical tissues (22, 23, 15-18, 12, 6-2) so that the root canal space does not turn into a source of infection (19). Reaching this is possible by removing diseased tissues and preventing re-infection (13, 24, 25). The dentist needs to have enough information and skills carry out do this (18). Mechanical use of devices alone cannot effectively clean the complex tubular network of the canal (5, 7, 12, 23). With the current methods of preparation, 40-50% of the wall surface of the canal remains intact and thus enough value of infected tissue remains so that microorganisms can survive and regrow (19). Hence, using irrigants along mechanical preparation is needed (12, 14, 16, 23). Using mechanical devices besides non-antimicrobial irrigants could diminish the bacteria in the root canal by up to 50% (14).

The significance of using irrigants in non-surgical endodontic therapy has often been neglected in the education of dental students and later in clinical practice (23). Disinfection of the root canal system by preparation and using irrigants is the key to reducing the number of bacteria from the root canal and helping to control periapical diseases (18). These materials complete mechanical debridement by flushing out debris, dissolving necrotic tissue, smearing, and disinfecting the root canal system (2,8,24). No single solution could completely do all this (24). Studies have revealed that the usual methods of using devices during the cleaning and shaping of a smear produce a coating that covers the walls of the canal and the entrance to the dentin tubules (1,9,13). This layer, with a diameter of 1-2 mµ, amorphous and irregular (3), is composed of organic matter of pulp and inorganic dentin debris, microorganisms, their products, and necrotic materials (1,3,6,9,13). The presence of smear layer prevents the penetration of drugs into the canal system into the root canal system and dentin tubules and prevents the complete adaptation of the filler material to the surface of the prepared canal walls (9,13).

Different acids, ultrasonic devices and lasers are used to remove this layer (13,9). One of the materials used for this purpose is using ethylenediaminetetraacetic acid (EDTA). This material is chelator able to remove inorganic components but not organic matter. Thus, using a tissue solvent such as sodium hypochlorite is necessary after that (1). Mixture of Tetracycline, Acid and Irrigant (MTAD) has proven effective in removing smear layer. Moreover, tetracycline is recommended as a chelator in endodontic therapy (13). Root canal irrigation is an important step during endodontics. There is no evidence that the outcome and success of treatment depend on the type of irrigant. Thus, there is no agreement on which irrigant is best, or whether it is better to use one irrigant or to use them at the same time. However, the agreement is agreed that the irrigant must have antimicrobial activity. The best results can be expected from an irrigant with good antimicrobial activity that has been in contact with the bacteria in the root canal system for a long time. This means that an enough volume of irrigant solution is needed during endodontics to complete the preparation stage before obturation (18). It seems that using topical medications is better and more effective than antibiotic applications (15). The purpose of the study was to examine cellular and tissue cytotoxicity of modified chlorhexidine an intracanal irrigant in vitro and ex-vivo.

MATERIALS AND METHODS

The population was apparently healthy adult Wistar female rats weighing g 200 grams. Sixteen rats were obtained from Laboratory Animal Reproduction and Breeding Center of Jundishapur University and examined for 90 days as follows. Rats were weighed prior to anesthesia to know their weight and calculate the dose of anesthetic. Firstly, the animals were anesthetized with ketamine and xylazine at doses of 100 and 5 mg / kg body weight, respectively. The animal back hair was shaved and the skin was disinfected with betadine solution. Then incisions (3 incisions) were made in the direction of the head back using Bistouri 15 along 2 cm. Each slice was at least 2 cm apart to prevent material interference. Three incisions were made in the back of each animal. Each of the three test materials was placed under sterile conditions in polyethylene tubes and placed in each of the cutting sites. Ultimately, the muscle and skin layers of all 3 incisions were sutured using 0.3 silica sutures. The animals were rested for one week and the stitches were removed on day 7 and ready-made pellets were used for feeding. Then the histological response to each of these substances was examined at 7, 15, 30, and 60-day intervals, and four rats were sacrificed in each of the mentioned

periods. To do this, euthanasia was carried out using chloroform. The skin of the tubular area was then sampled.

After cutting the skin, the skin pieces were attached to cardboard paper with a pin to prevent the skin from shrinking. The samples were placed in a volume of at least 10 times of 10% formalin saline for stabilization, and were transferred to the pathology laboratory of Ahwaz Veterinary School after recording the necessary specifications, such as sample number and sampling date, on the containers containing the sample. The stabilized samples were prepared by standard method of tissue sections for microscopic studies. In this method, various stages of passage such as dewatering, clarification and paraffin impregnation were performed using Histokinet 2000 after fixing and washing with running water. Then the samples were molded and sections with a thickness of 5-6 μm were prepared using a rotary microtome, and stained using hematoxylin-eosin (H&E) methods. Hematoxylin-eosin staining is the most common and widely used staining in histology. In this staining, the cell nucleus turns purple and the cytoplasm turns pink. The method of preparation of colors and staining steps were performed by conventional methods. The hematoxylin used in this study was H&E was alcoholic. Changes in the number of various inflammatory cells (lymphocytes, plasma cells, PMN, macrophages and giant cells) were examined and counted by light microscope. Three microscopic fields from each slide and five slides from each group were randomly selected and examined with a 40 light microscope lens to count the cells.

At in vitro stage, gingival fibroblasts were prepared from Pasteur Institute Cell Bank of Tehran and were amplified in 25cm^2 flasks at 37 ° C in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS) and antibiotics (penicillin (100 U / ml)) and streptomycin (0.1 mg / ml). and antifungals (amphotericin B (1.25 μg / ml)) until the experiment was performed. The flasks were incubated at 37 ° C in the presence of 2.5% CO_2 and 88% humidity. The culture medium was changed every other day until the maximum growth of fibroblasts was reached and then the cells were isolated with EDTA trypsin 0.25% and were transferred to a new flask after counting using a hemocytometer. Then 96-well plates were filled with 50 μl of culture medium containing 20,000 fibroblast cells and placed in a greenhouse cell culture incubator for 24 hours. The following day the culture medium was discarded and all plates were washed with phosphate-buffered saline (PBS). It must be stated that all stages of the experiment were performed under the hood to prevent contamination of the samples. The test was performed as triplicate (presence of 3-well cell) (12).

The degree of cytotoxicity in this experiment was demarcated using formason composed of tetrazolium yellow salt crystals affected by lactate dehydrogenase (LDH) enzyme, released only from the cytoplasm of lysed cells, and mitochondrial succinate dehydrogenase, active only in living cells and were measured using determination of adsorption by ELISA method by ELISA reader at 490 and 492 nm wavelengths, respectively (using LDH Plus kit with serial number 11644793001 made by Roche, Germany). Besides the experimental groups, 3 other groups, including high control background and low control were needed to measure toxicity using LDH. Thus, only the culture medium was considered in the background group, lysis in the kit (white cap) to completely release LDH and complete lysis of the cells in the high control group, and the cells were placed in the low control group. In the experimental groups, after placing each irrigant solution adjacent to the cells in the incubator for 15 minutes, 100 μl of the reaction mixture was added to the cells and placed in the incubator again for 30 minutes. Then 50 μl of stop solution was added to it and the absorbance was measured after shaking for 10 seconds. Quantitative data was expressed in SPSS-16 as mean ± standard error of the mean (SEM). ANOVA ($P<0.05$) was used as a statistically significant level to determine the differences between the groups.

RESULTS

In the statistical analyses in software, sig (p-value) (p-value) is calculated. We compare the value of sig with the significant level of the test (for example, 5%). If it is smaller, the null hypothesis is rejected and the alterative hypothesis is accepted. In this study, one-way ANOVA and LSD statistical tests were used for pairwise comparison of the data. The mean cytotoxicity of the tested data is given in Table 1.

Table 1. Mean cytotoxicity of various groups

Groups	Frequency	Mean	Standard deviation	Standard error	95% confidence interval		Min.	Max.
					Low band	High band		
Modified chlorhexidine in 2 Landa scale	4	27.50	14.271	7.136	4.79	50.21	11	43
Modified chlorhexidine in 4 Landa scale	4	17.75	9.912	4.956	1.98	33.52	5	27
2% chlorhexidine in 2 Landa scale	4	56.00	7.832	3.916	43.54	68.46	48	66
2% chlorhexidine in 4 Landa scale	4	50.50	19.672	9.836	19.20	81.80	25	70
Sodium hypochlorite 2.5% in 2 Landa scale	4	69.25	8.180	4.090	56.23	82.27	58	77
Sodium hypochlorite 2.5% in 4 Landa scale	4	23.50	15.716	7.858	-1.51	48.51	7	44
Sodium hypochlorite 5.25% in 2 Landa scale	4	41.75	38.135	19.067	-18.93	102.43	5	94
Sodium hypochlorite 5.25% in 4 Landa scale	4	53.00	36.414	18.207	-4.94	110.94	5	88
Total	32	42.41	25.789	4.559	33.11	51.70	5	94

Statistical examinations indicated a significant difference between the 8 groups examined for cytotoxicity (P = 0.033) (Table 2).

Table 2. Statistical examination of cytotoxicity of various groups using ANOVA test

	Sum of squares	**Degree of freedom**	**Mean square**	**F**	**Sig.**
Among 8 groups	9084.469	7	1297.781	2.701	.033

The examination using ANOVA and Tukey test (HSD) test showed a significant difference in cytotoxicity between modified chlorhexidine in Landa scale 4 and 2.5% sodium hypochlorite in Landa scale 2 (P = 0.049).

Table 3. Comparison of cytotoxicity of various groups

Group (I)	**Group (J)**	**Mean difference (I-J)**	**Standard error**	**Sig.**	**%95 confidence interval**	
					Low band	**High band**
Modified chlorhexidine on a Landa2 scale	Modified chlorhexidine in 4 Landa scale	9.750	15.501	.998	-41.59	61.09
	2% chlorhexidine in 2 Landa scale	-28.500	15.501	.602	-79.84	22.84
	2% chlorhexidine in 4 Landa scale	-23.000	15.501	.808	-74.34	28.34
	Sodium hypochlorite 2.5% in 2 Landa scale	-41.750	15.501	.173	-93.09	9.59
	Sodium hypochlorite 2.5% in 4 Landa scale	4.000	15.501	1.000	-47.34	55.34
	Sodium hypochlorite 5.25% in 2 Landa scale	-14.250	15.501	.981	-65.59	37.09
	Sodium hypochlorite 5.25% in 4 Landa scale	-25.500	15.501	.720	-76.84	25.84
Modified chlorhexidine in 4 Landa scale	Modified chlorhexidine in 2 Landa scale	-9.750	15.501	.998	-61.09	41.59
	2% chlorhexidine in 2 Landa scale	-38.250	15.501	.256	-89.59	13.09
	2% chlorhexidine in 4 Landa scale	-32.750	15.501	.435	-84.09	18.59
	Sodium hypochlorite 2.5% in 2 Landa scale	-51.500*	15.501	**.049**	-102.84	-.16
	Sodium hypochlorite 2.5% in 4 Landa scale	-5.750	15.501	1.000	-57.09	45.59
	Sodium hypochlorite 5.25% in 2 Landa scale	-24.000	15.501	.774	-75.34	27.34
	Sodium hypochlorite 5.25% in 4 Landa scale	-35.250	15.501	.347	-86.59	16.09
2% chlorhexidine in 2 Landa scale	Modified chlorhexidine in 2 Landa scale	28.500	15.501	.602	-22.84	79.84
	Modified chlorhexidine in 4 Landa scale	38.250	15.501	.256	-13.09	89.59
	2% chlorhexidine in 4 Landa scale	5.500	15.501	1.000	-45.84	56.84
	Sodium hypochlorite 2.5% in 2 Landa scale	-13.250	15.501	.987	-64.59	38.09
	Sodium hypochlorite 2.5% in 4 Landa scale	32.500	15.501	.444	-18.84	83.84
	Sodium hypochlorite 5.25% in 2 Landa scale	14.250	15.501	.981	-37.09	65.59
	Sodium hypochlorite 5.25% in 4 Landa scale	3.000	15.501	1.000	-48.34	54.34
2% chlorhexidine in 4 Landa scale	Modified chlorhexidine in 2 Landa scale	23.000	15.501	.808	-28.34	74.34
	Modified chlorhexidine in 4 Landa scale	32.750	15.501	.435	-18.59	84.09
	2% chlorhexidine in 2 Landa scale	-5.500	15.501	1.000	-56.84	45.84
	Sodium hypochlorite 2.5% in 2 Landa scale	-18.750	15.501	.921	-70.09	32.59
	Sodium hypochlorite 2.5% in 4 Landa scale	27.000	15.501	.662	-24.34	78.34
	Sodium hypochlorite 5.25% in 2 Landa scale	8.750	15.501	.999	-42.59	60.09
	Sodium hypochlorite 5.25% in 4 Landa scale	-2.500	15.501	1.000	-53.84	48.84

	Modified chlorhexidine in 2 Landa scale	41.750	15.501	.173	-9.59	93.09
	Modified chlorhexidine in 4 Landa scale	51.500*	15.501	**.049**	.16	102.84
	2% chlorhexidine in 2 Landa scale	13.250	15.501	.987	-38.09	64.59
	2% chlorhexidine in 4 Landa scale	18.750	15.501	.921	-32.59	70.09
	Sodium hypochlorite 2.5% in 4 Landa scale	45.750	15.501	.106	-5.59	97.09
	Sodium hypochlorite 5.25% in 2 Landa scale	27.500	15.501	.642	-23.84	78.84
	Sodium hypochlorite 5.25% in 4 Landa scale	16.250	15.501	.961	-35.09	67.59
Sodium hypochlorite 2.5% in 2 Landa scale	Modified chlorhexidine in 2 Landa scale	-4.000	15.501	1.000	-55.34	47.34
	Modified chlorhexidine in 4 Landa scale	5.750	15.501	1.000	-45.59	57.09
	2% chlorhexidine in 2 Landa scale	-32.500	15.501	.444	-83.84	18.84
	2% chlorhexidine in 4 Landa scale	-27.000	15.501	.662	-78.34	24.34
	Sodium hypochlorite 2.5% in 2 Landa scale	-45.750	15.501	.106	-97.09	5.59
	Sodium hypochlorite 5.25% in 2 Landa scale	-18.250	15.501	.931	-69.59	33.09
	Sodium hypochlorite 5.25% in 4 Landa scale	-29.500	15.501	.562	-80.84	21.84
Sodium hypochlorite 2.5% in 4 Landa scale	Modified chlorhexidine in 2 Landa scale	14.250	15.501	.981	-37.09	65.59
	Modified chlorhexidine in 4 Landa scale	24.000	15.501	.774	-27.34	75.34
	2% chlorhexidine in 2 Landa scale	-14.250	15.501	.981	-65.59	37.09
	2% chlorhexidine in 4 Landa scale	-8.750	15.501	.999	-60.09	42.59
	Sodium hypochlorite 2.5% in 2 Landa scale	-27.500	15.501	.642	-78.84	23.84
	Sodium hypochlorite 2.5% in 4 Landa scale	18.250	15.501	.931	-33.09	69.59
	Sodium hypochlorite 5.25% in 4 Landa scale	-11.250	15.501	.995	-62.59	40.09
	Modified chlorhexidine in 2 Landa scale	25.500	15.501	.720	-25.84	76.84
	Modified chlorhexidine in 4 Landa scale	35.250	15.501	.347	-16.09	86.59
	2% chlorhexidine in 2 Landa scale	-3.000	15.501	1.000	-54.34	48.34
	2% chlorhexidine in 4 Landa scale	2.500	15.501	1.000	-48.84	53.84
	Sodium hypochlorite 2.5% in 2 Landa scale	-16.250	15.501	.961	-67.59	35.09
	Sodium hypochlorite 2.5% in 4 Landa scale	29.500	15.501	.562	-21.84	80.84
	Sodium hypochlorite 5.25% in 2 Landa scale	11.250	15.501	.995	-40.09	62.59

* The average difference is significant at 0.05.

No significant macroscopic changes were seen in the female rats examined during the 60-day experimental period and administration of the drugs. On the day of the experiment, the rats receiving sodium hypochlorite received 25.5% showed a rapid and severe tissue reaction as darkening of the site, but was resolved after 7 days. No reaction was observed in other groups. No differences in position were observed after finishing the experimental period of rat euthanasia and sampling the studied organ and its appearance and macroscopic examination.

In microscopic examination of skin tissue sections, the skin structure of rats of various groups was examined with a light microscope using the usual H&E staining method. The tissue changes seen in various groups were described and compared using microscopic images. In the group receiving 25.5% sodium hypochlorite, histological response was observed on days 7, 15, 30, 60. The number of inflammatory cells had increased in this group compared to other groups. In some samples, an increase in abnormal (deformed) nuclei, accompanied by accumulation of inflammatory cells, infiltration of lymphocyte cells into the subcutaneous space was observed throughout the studied lamellae, which were seen as destroyed cytoplasmic limits of skin cells and changes in the nuclei such as fragmentation, shrinkage and clear spaces in the cytoplasm, the existence of single-celled inflammatory cells, and the infiltration of lymphocytes under the skin. These conditions were not seen in the samples of the 60th day and a mild tissue reaction was observed.

In the group receiving 2% chlorhexidine, the number of abnormal cells had reduced compared to the sodium hypochlorite group. Additionally, the increase of inflammatory cells along with the penetration of

mononuclear cells into the subcutaneous spaces in this group was important.

In some samples, an increase in abnormal (deformed) nuclei, accompanied by accumulation of inflammatory cells, infiltration of lymphocyte cells into the subcutaneous space was observed throughout the studied lamellae, which were seen as destroyed cytoplasmic limits of skin cells and changes in the nuclei such as fragmentation, shrinkage and clear spaces in the cytoplasm, the existence of single-celled inflammatory cells, and the infiltration of lymphocytes under the skin. These conditions were not seen in the samples of days 30 and 60 and a very mild tissue reaction was observed. In the group receiving modified chlorhexidine that received modified chlorhexidine, the skin cell changes were less than in the previous groups. Furthermore, the accumulation of inflammatory cells was less than the previous groups. In the samples of days 15, 30, these conditions were not seen and a mild tissue reaction was seen, and the condition was not seen on day 60 and a mild tissue reaction was seen only.

In micrometric analysis, the results obtained in the study are given in (Table 1). According to the results, most of the scores obtained are in the first and second periods of the experiment showing the presence of moderate to severe inflammation in the samples with mild inflammation in the third and fourth periods. On day 7, inflammation in 2% chlorhexidine and 25.5% sodium hypochlorite was severe and severe to very severe, but in modified chlorhexidine the inflammation was moderate to severe. On day 15, modified chlorhexidine and 2% chlorhexidine inflammation was normal and sodium hypochlorite was 25.5% severe inflammation. On day 30, inflammation in 2% chlorhexidine and modified chlorhexidine was mild to moderate and mild, and in sodium hypochlorite 5.25% inflammation was moderate to severe. On day 60, modified chlorhexidine was very mild, chlorhexidine was 2% mild, and sodium hypochlorite 5.25% was mild to moderate. Overall, it is shown that inflammation decreases over time in all samples.

Table 4. Total results obtained in all intracanal irrigants in all periods in Robbins scoring from 0-4

Intracanal irrigants	Periods			
	Day 7	Day 15	Day 30	Day 60
Modified chlorhexidine	3 3 3 2	3 2 2 2	1 2 2 1	1 1 1 0
Mean	2.75	2.25	1.5	0.75
2% chlorhexidine	4 3 3 2	2 2 3 3	1 2 3 1	1 1 2 1
Mean	3	2.5	1.75	1.25
Sodium hypochlorite 5.25%	4 4 3 4	3 3 4 3	3 2 3 3	2 1 2 2
Mean	3.75	3.25	2.75	1.75

In the following table of the Kruskal-Wallis test, the value of Chi-Square (9.089) is significant at the error level less than 0.01. Hence, there is a statistically significant difference in the rate of cellular and tissue cytotoxicity in intracanal irrigants (P = 0.011).

Table 5. Comparison of all intracanal irrigants

	Inflammation	Group	Frequency	Rank mean
Chi-Square	9.089	Modified chlorhexidine	16	18.63
Degree of freedom	2	2% chlorhexidine	16	22.41
Asymp. Sig.	.011	Sodium hypochlorite	16	32.47
		Total	48	

The table below is the results of the Mann-Whitney test with reference to various z values of Z. Statistically, the degree of cellular and tissue cytotoxicity was not significantly different between modified chlorhexidine and 2% chlorhexidine (P= 0.417). However, the performance rank of modified chlorhexidine (15.22) is higher than 2% chlorhexidine (17.78). Thus, modified chlorhexidine has less toxicity and inflammation than 2% chlorhexidine. Statistically with 0.95 confidence the degree of cellular and tissue cytotoxicity between chlorhexidine and sodium hypochlorite was statistically different (P = 0.004). The performance rank of modified chlorhexidine (11.91) is higher than that of sodium hypochlorite (21.09). Hence, modified chlorhexidine has much less toxicity and inflammation than sodium hypochlorite. Statistically with 0.95 confidence, the degree of cytological and histological toxicity is different between 2% chlorhexidine and sodium hypochlorite (P = 0.033). The performance rank of 2% chlorhexidine is (13.13) higher than sodium hypochlorite (19.88). Hence, 2% chlorhexidine has much less toxicity and inflammation than sodium hypochlorite.

Table 6. Pairwise comparison of the intracanal irrigants with each other

Statistical test	Inflammation		Intracanal irrigants	Frequency	Rank mean	Rank total
Mann-Whitney U	107.500	Reference to Z value (0.811)				
Wilcoxon W	243.500					
Z	-.811	Group	Modified chlorhexidine			
Asymp. Sig. (2-tailed)	.417		2% chlorhexidine			
Exact Sig. [2*(1-tailed Sig.)]	.445[b]		Total			
Mann-Whitney U	54.500	Reference to Z value (2.886)				
Wilcoxon W	190.500					
Z	-2.886	Group	Modified chlorhexidine			
Asymp. Sig. (2-tailed)	.004		Sodium hypochlorite			
Exact Sig. [2*(1-tailed Sig.)]	.004[b]		Total			
Mann-Whitney U	74.000	Reference to Z value (2.127)				
Wilcoxon W	210.000					
Z	-2.127	Group	2% chlorhexidine			
Asymp. Sig. (2-tailed)	.033		Sodium hypochlorite			
Exact Sig. [2*(1-tailed Sig.)]	.043[b]		Total			

The table below from the Kruskal-Wallis test indicated that the Chi-Square values on day 7 (4.446), day 15 (5.083), day 30 (4.813) and day 60 (5.263) were insignificant at the error level less than 0.01. Hence, statistically, the rate of cellular and tissue cytotoxicity in intracanal irrigants was not significantly different on day 7 (P = 0.108), day 15 (P = 0.079), day 30 (P = 0.090) and day 60 (P = 0.072). Therefore, in none of the time periods is there a significant difference between the intracanal irrigants.

Table 7. Comparison of all intracanal irrigants at various periods

Day	Inflammation		Group	Frequency	Rank mean
Day 7	Chi-Square	4.446	Modified chlorhexidine	4	4.50
	df	2	2% chlorhexidine	4	5.75
	Asymp. Sig.	.108	Sodium hypochlorite	4	9.25
			Total	12	
Day 15	Chi-Square	5.083	Modified chlorhexidine	4	4.38
	df	2	2% chlorhexidine	4	5.75
	Asymp. Sig.	.079	Sodium hypochlorite	4	9.38
			Total	12	
Day 30	Chi-Square	4.813	Modified chlorhexidine	4	4.50
	df	2	2% chlorhexidine	4	5.50
	Asymp. Sig.	.090	Sodium hypochlorite	4	9.50
			Total	12	
Day 60	Chi-Square	5.263	Modified chlorhexidine	4	4.00
	df	2	2% chlorhexidine	4	6.38
	Asymp. Sig.	.072	Sodium hypochlorite	4	9.13
			Total	12	

In the table below of the Kruskal-Wallis test, the value of Chi-Square (24.736) is significant at the error level less than 0.01. Hence, statistically, the rate of cellular and tissue cytotoxicity of intracanal irrigants in different periods is significantly different (P = 0.0001).

Table 8. Comparison of different periods

Inflammation		Day		Frequency	Rank mean
Chi-Square	24.736		Day 7	12	36.42
df	3		Day 15	12	29.92
Asymp. Sig.	.000		Day 30	12	20.83
			Day 60	12	10.83
			Total	48	

The table below is the results of the Mann-Whitney test with reference to different Z values. Statistically, the rate of cellular and tissue cytotoxicity of intracanal irrigants, with 0.95 confidence, was not different between day 7 and day 15 (P = 0.089). The performance rank of day 15 (10.25) is higher than day 7 (14.75). Thus, the toxicity and inflammation of intracanal irrigants on day 15 are much less than on day 7. Statistically, the degree of cellular and tissue cytotoxicity of intracanal irrigants, with 0.95 confidence, varied between day 7 and day 30 (P = 0.004). The performance rank of day 30 (8.50) is higher than day 7 (16.50). Thus, the toxicity and inflammation of intracanal irrigants on day 30 are much less than on day 7.

Table 9. Pairwise comparison of two periods with each other

Statistical test	Inflammation		Intracanal irrigants	Frequency	Rank mean	Rank total
Mann-Whitney U	45.000	Reference to Z value (1.698)				
Wilcoxon W	123.000					
Z	-1.698	Group	7 days	12	14.75	177.00
Asymp. Sig. (2-tailed)	.089		15 days	12	10.25	123.00
Exact Sig. [2*(1-tailed Sig.)]	.128[b]		Total	24		
Mann-Whitney U	24.000	Reference to Z value (2.914)				
Wilcoxon W	102.000					
Z	-2.914	Group	7 days	12	16.50	198.00
Asymp. Sig. (2-tailed)	.004		30 days	12	8.50	102.00
Exact Sig. [2*(1-tailed Sig.)]	.005[b]		Total	24		
Mann-Whitney U	4.000	Reference to Z value (4.047)				
Wilcoxon W	82.000					
Z	-4.047	Group	7 days	12	18.17	218.00
Asymp. Sig. (2-tailed)	.000		60 days	12	6.83	82.00
Exact Sig. [2*(1-tailed Sig.)]	.000[b]		Total	24		
Mann-Whitney U	42.000	Reference to Z value (1.885)				
Wilcoxon W	120.000					
Z	-1.855	Group	15 days	12	15.00	180.00
Asymp. Sig. (2-tailed)	.064		30 days	12	10.00	120.00
Exact Sig. [2*(1-tailed Sig.)]	.089[b]		Total	24		
Mann-Whitney U	10.000	Reference to Z value (3.756)				
Wilcoxon W	88.000					
Z	-3.756	Group	15 days	12	17.67	212.00
Asymp. Sig. (2-tailed)	.000		60 days	12	7.33	88.00
Exact Sig. [2*(1-tailed Sig.)]	.000[b]		Total	24		
Mann-Whitney U	38.000	Reference to Z value (2.112)				
Wilcoxon W	116.000					
Z	-2.112	Group	30 days	12	15.33	184.00
Asymp. Sig. (2-tailed)	.035		60 days	12	9.67	116.00
Exact Sig. [2*(1-tailed Sig.)]	.052[b]		Total	24		

Statistically, the value of cellular and tissue cytotoxicity of intracanal detergents varied between day 7 and day 60 (P = 0.0001), with a confidence of 0.95. The performance rank of day 60 (6.83) is higher than day 7 (18.17). Thus, the toxicity and inflammation of intracanal irrigants on day 60 are much less than on day 7. Statistically, the value of cellular and tissue cytotoxicity of intracanal irrigants did not differ between day 15 and day 30 (P = 0.064), with a confidence of 0.95. The performance rank of day 30 (10.00) is higher than day 15 (15.00). Therefore, the toxicity and inflammation of intracanal irrigants on day 30 are much less than on day 15.

Statistically, the value of cellular and tissue cytotoxicity of intracanal detergents varied between day 15 and day 60 (P = 0.000), with a confidence of 0.95. The performance rank of day 60 (7.33) is higher than day 15 (17.67). Therefore, the toxicity and inflammation of intracanal irrigants on day 60 are much less than on day 15. Statistically, the value of cellular and tissue cytotoxicity of intracanal detergents varied between day 30 and day 60 (P = 0.035), with a

confidence of 0.95. The performance rank of day 60 (9.67) is higher than day 30 (15.33). Therefore, the toxicity and inflammation of intracanal irrigants on day 60 are much less than on day 30.

In the table below of Kruskal-Wallis test, Chi square value (9.089) at the error level less than 0.01 is significant. Thus, the value of cellular and tissue cytotoxicity of intracanal detergents was significantly different (P=0.011).

Table 10. Comparison of all intracanal irrigants

Group	Frequency	Rank mean		Inflammation
Modified chlorhexidine	16	18.63	Chi square	9.089
2% chlorhexidine	16	22.41		
Sodium hypochlorite	16	32.47	Degree of freedom	2
total	48		Asymp. Sig.	0.011

Discussion

Examining the results of this study showed a significant difference between the tissue reactions in various materials (P. Value <0.05). Furthermore, most of the scores created in the first, second and third periods in all materials are about 2 and 3 and in the next period between 1 and 2. Percentage of primary inflammation is probably due to trauma from surgery. Watts et al. (26) stated that chlorhexidine exerts a mild chemotaxis on neutrophils at low concentrations and mobilizes neutrophils at high concentrations. This is in line with the results of the previous studies where low concentrations of chlorhexidine lead to a slight increase in neutrophil transport, whereas higher concentrations of this substance reduce this transport. Nonetheless, other studies have reported a greater stimulus potential for chlorhexidine. Kenney et al. (27) examined the effect of chlorhexidine on multinucleated leukocytes showing that 0.2% concentration caused severe degradation in these cells.

Goldschmidt et al. (28) concluded that chlorhexidine can be toxic even at low concentrations such as 0.001 and 0.02. Although the number of inflammatory cells was high on day 7 in this study, it was significantly reduced on days 15 and 30. A similar result was seen on day 14 in other studies that had rated chlorhexidine 0.12% as an irrigant. These studies had the highest inflammatory response at 48 hours, which decreased at the end of the second week (29, 30). Turkun et al. (29) claimed that chlorhexidine has 1% less toxicity, less degradation and faster repair than 0.5% sodium hypochlorite, which is in line with the results of this study. Turkun also reported that chlorhexidine has no less antibacterial effect than sodium hypochlorite and may be preferred for root canal treatment because of its low toxicity, especially in teeth with open apex.

In this study, sodium hypochlorite showed a 5.25% more severe inflammatory response until day 30. This is very similar to the results of other studies that reported a severe inflammatory reaction until day 14. (31). Yesilsoy et al. (30) reported that 5% sodium hypochlorite had a stimulatory effect on periapical tissue and were formed by the end of the second week of foreign body granuloma evaluation. As in the present study, other studies have reported that the number of inflammatory cells in the areas of contact with sodium hypochlorite was 5.25% that increased until day 15 (32-39). Oncag et al. (32) stated that 2% chlorhexidine has extracellular antibacterial properties and is less toxic than 5.25% sodium hypochlorite. Hidalgo et al. indicated the destruction of fibroblasts at high concentrations of chlorhexidine 0001% (33). Sanches et al. (1988) showed that chlorhexidine at concentrations of 0.5 and 0.005% had toxic effects on fibroblasts. Tatnal et al. found that chlorhexidine has a toxic effect on keratinocytes (34). Damour et al. (1992) and Fabreguette (1994) showed the toxic effects of chlorhexidine on fibroblasts and keratinocytes (35).

Boyce et al. (1995) reported that chlorhexidine at a concentration of 0.05% had both a toxic effect on fibroblasts and keratinocytes and killed microorganisms at this concentration. Chang et al. indicated the toxic effect of chlorhexidine on PDL cells (36). Nakamura and Iwasawa (2003) reported the toxic effects of chlorhexidine at concentrations of 0.0004 and 0.0002% on epidermal cells (37). Ciancio et al. stated that the minimum concentration of chlorhexidine, which had toxic effects on fibroblasts, was 0.002% (38). On the other hand, Mariotti et al reported a concentration of 0.0009%. The reason for the discrepancy between the studies can be the difference in the time of exposure of cells to chlorhexidine, the incubation time of cells, the difference in the test used to determine the percentage of live cells and the difference in the percentage of fetal calf serum (FCS) used in culture (chlorhexidine binding to protein of serum and its effective dose reduction in the vicinity of cells) (39). Pucher et al. (1997) examined the effect of different concentrations of chlorhexidine on fibroblast cells and concluded that 0.002% had the lowest toxicity effect on fibroblast cells (40). Yu-chao Chang et al. (2001) studied the effect of chlorhexidine and sodium hypochlorite on periodontal ligament cells. Concentrations 0.025%, 0.05%, 0.2% and 4% sodium hypochlorite, 0.001%, 0.001%, 0.01% and chlorhexidine on periodontal cells were evaluated for their effect on protein synthesis and mitochondrial activity. The experiment found that sodium and chlorhexidine hypochlorite have a toxic effect on cells, whereas CHX inhibits protein synthesis and sodium hypochlorite has no such effects. Moreover, CHX and sodium hypochlorite have an inhibitory effect on

mitochondrial activity (41). The toxicity of chlorhexidine and sodium hypochlorite is in line with the present study, although the cells used in this study vary.

Barnhart et al. (2005) examined the toxicity of sodium hypochlorite, potassium iodide, calcium hydroxide, and chlorine dioxide on fibroblast cells. The analysis was carried out by logarithmic difference. IKI and Ca (OH)2 were less toxic than SCD, NAOCL and betadine. LKL and Ca(OH)2 are easily tolerated by fibroblasts (42). The higher toxicity of hypochlorite than other tested substances is in line with the current study. Yu-chao De Souza LB et al. (2007) studied the effect of various concentrations of chlorhexidine and hydrogen peroxide on odontoblast-like cells. This effect was measured by measuring cellular metabolic activity through MTT Assay and cell morphology through SEM. The results indicated that CHX 0.02% had a highly toxic effect on odontoblast-like cells, whereas chlorhexidine 0.004% and 0.0024% had toxic effects on cells (43). As in the present study, Giannelli et al. (2007) studied the effect of chlorhexidine on osteoblast, fibroblast, and endothelial cells. The findings indicated that CHX showed its toxic effect in a dose-dependent and time-dependent way, especially on osteoblast cells (44). The reason for the discrepancy between the studies can be the difference in the time of exposure of cells to chlorhexidine, the incubation time of cells, the difference in the test used to specify the percentage of live cells and the difference in the percentage of FCS used in culture medium (chlorhexidine binding to serum protein and reduction in its effective dose in the vicinity of cells).

Conclusion

A glance at the results of these studies, it is realized that common irrigants such as sodium hypochlorite and chlorhexidine have significant toxicity to biological cells. The toxicity of modified chlorhexidine relative to sodium hypochlorite was 5.25% in ex-vivo study and 2.5% to sodium hypochlorite in in-vitro study. One can perceive that this substance is not more toxic than other endodontic irrigants. The toxicity results of modified chlorhexidine solution cannot be compared to the results of other articles as it is a completely new substance and calls for further and more extensive studies in the future papers.

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