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Original Article

Comparison of the permeability rate of nanoparticle calcium hydroxide and conventional calcium hydroxide using a fluorescence microscope

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ABSTRACT

Background: The permeability feature of conventional calcium hydroxide (CH) and nanoparticle CH (NCH) was compared to show the desired effects of this new material and in case of confirmation of its other properties; CH can be used as a safe alternative.

Materials and Methods: This *in vitro* was carried out in two phases: First phase: measurement and comparison of the permeability rate of conventional CH and NCH in the dentinal tubules employing a fluorescence microscope were carried out. Second phase: measurement and comparison of the permeability rate of NCH and conventional CH in L929 fibroblast cells using a fluorescence microscope were carried out. Kruskal–Wallis analysis was used for overall comparisons. A series of Mann–Whitney U tests were used for pair-wise comparisons ($P < 0.05$).

Results: Based on the results of Kruskal–Wallis test, in all three regions of the cervical third, middle third, and apical third of the root, mean values of the percentage difference of the fluorescence and color change in NCH were more than the conventional CH, and the difference was statistically significant ($P < 0.001$). the percentage of fluorescence color change in drug with the concentration of 1 g/cc was more than the one with the concentration of 0.1 g/cc and the difference was statistically significant ($P < 0.001$).

Conclusion: The nanoparticle drug compared with the conventional drug has a more penetration depth in all regions of the root of the dentinal tubules.

Key Words: Calcium hydroxide, endodontics, fluorescence, nanoparticle, permeability

INTRODUCTION

Nowadays, there are still many cases of failure in root canal treatment despite great advances in the science of endodontics. Undoubtedly, the main cause of this failure is attributed to the fact that microorganisms remain within the root canal system.[1] Studies have shown that despite various methods of root canal preparation including a variety of mechanical and chemical cleaning methods, germ-free channel access is still difficult in the infected tooth and most of the microorganisms that remain within the dentinal tubules have the capability to replicate and disrupt the repairing process of periapical tissue.[2] Nevertheless, it should be noted that most bacteria of root canals are

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eliminated during root canal mechanical preparation, but because of the complexity of the root canal system, it is not possible to destroy the bacteria that penetrated into the dentinal tubules by employing only these methods.[3,4] That's why various materials are employed during canals preparation phase and in the interval between treatment sessions to destroy the remaining necrotic tissue and microorganisms that penetrated into the canal. Several drugs have been proposed for this purpose, but dentists have been using calcium hydroxide (CH) as the drug of choice between treatment sessions to the more effectively prepared root canal system.[5-9] The CH drug shows a pH of 12 and its hydroxyl ions lead to the destruction of bacterial cell walls; thus, this material demonstrates broad-spectrum antimicrobial effects.[10] This drug can dissolve tissue debris and prevents root surface atrophy.[11,12] Another study has also demonstrated that hydroxyl ion can enter the dentinal tubules and reaches the outer surface of the root within 7 days; therefore, it has been stated in most reference books that CH must stay as an inter-treatment session drug in the root canal for 7 days.[13] This drug has been made in various forms in recent years. Some pharmacists have made some changes in its appearance (dry powder, thick paste, syringe paste, and gel) and some of them have made various compounds of this drug; however, in the meantime, CH is generally supplied as a dry powder, which must be mixed with the anesthetic solution, normal saline, water, or glycerin until it is ready for use.[14,15] Since this drug must be in direct contact with microorganisms for sufficient efficacy, it must have the ability to penetrate the dentinal tubules because microorganisms such as *Enterococcus faecalis* can penetrate the tubules and protect themselves from the effects of this drug. However, Komabayashi *et al*. had demonstrated that many particles of conventional CH, due to large size of the particles, cannot ideally penetrate into the dentinal tubules.[16] On the other hand, nanotechnology has currently become one of the most important technologies in various fields of science and industry, and its applications in different fields enable us to carry out processes and thinking about which was too far-fetched in the past. Basically, construction and operation of particles smaller than 100 nm require the utilization of nanotechnology. After construction and quality control nanomaterial, we decided to evaluate the performance of new material, and finally, in this experimental-laboratory study, the permeability feature of conventional CH and nanoparticle CH (NCH) was compared *in vitro* to show the desired effects of this new material.

MATERIALS AND METHODS

In this *in vitro* study two evaluations were done: dentinal tubules penetration and L929 fibroblast cells penetration.

Dentinal tubules penetration

Measurement and comparison of the permeability rate of conventional CH and NCH in the dentinal tubules employing a fluorescence microscope were carried out. For this purpose, 16 human single-rooted premolar adult teeth, with no cavities and crack and root curvature and resorption, were selected. The absence of calcification and single-rooted teeth was confirmed by radiography. Teeth were cleaned utilizing the cavitron device and were placed in 5.25% sodium hypochlorite (Taj, Iran) for half an hour for disinfecting and then placed in the normal saline solution for half an hour. The crowns were cut from the cementum-enamel junction part using the two-sided diamond disc (Jota, Swiss). Root length was calculated by file number 15 (Mani, Japan) (when the file tip overlapped the end part of the root with a 1-mm reduction from it) and root canal widening was done up to the file number 80 (Mani, Japan), using step-back technique. Washing operation was carried out using 5 mL of 2.5% sodium hypochlorite between each two file. Thereafter, smear layer was removed through washing with 5 mL of 5.25% sodium hypochlorite for 3 min and then 5 mL of 17% ethylenediamine tetraacetic acid (Merck, UK) for 3 min, as well as with 5 mL of distilled water.[17] The canals were dried using Paper Cone No. 30 (GAPA, Germany). One gram of conventional CH (Merck, UK) labeled with tetracycline and 1 gram (g) of NCH labeled with tetracycline were mixed with 1 mililiter (cc) serum. NCH was recently synthesized for dental applications as described earlier by Roy and Bhattacharya[18] with some modifications. 8 Teeth's canals were filled using conventional CH labeled with tetracycline and other 8 Teeth's canals were filled using NCH labeled with tetracycline, and the canal orifices were sealed with light-cure composite resin (3M, USA). Teeth were placed in an incubator under conditions of 37°C and humidity of 100% for 1 week. After a week, teeth were cut using Mecatome (Presi, France) with a thickness of 0.3 mm (Water of microtome only can wash surface layer of the drugs on teeth). Each tooth

was cut nine times (three cuts in the cervical one-third, three cuts in the middle third, and three cuts in the apical third). A total of 144 samples were prepared. The direction of cuts was as follows: eight teeth were cut perpendicular to the axis of the root and the other 8 teeth with an angle of 30° to the axis of the root (to view the dentinal tubules in different directions). The samples were analyzed utilizing the RX50 fluorescence microscope (Labex, UK). Finally, depth of penetration of particles between the canal and the outer surface roots was recorded using XSA1015-TG fluorescence spectra analyzer (Owon, Hong Kong). For this purpose, the percentage of fluorescence color change on the teeth walls and the outer surface of the root canal in three points of each sample was measured, and the average difference between any two points was recorded. The percentage of fluorescence color was measured with "Lum" parameter of colors in Paint software [Figure 1].

L929 fibroblast cells penetration

Measurement and comparison of permeability rate of NCH and conventional CH in L929 fibroblast cells using a fluorescence microscope were carried out. For this purpose, L929 cells (Pasteur Institute, Iran) were cultured pending the attainment of the intended amount of 500 mL. Subsequently, some medium (GIBCO, Grand Island New York, U. S) was added to them. After several times of changing the medium, the contents of the tubes were poured into the flask and were placed inside the incubator at 37°C, 98% humidity, and 5% CO_2. The contents of the flask were removed after 3–4 h to remove the cells unattached to the bottom of the flask. When the flask was removed from the incubator, and its liquid was discarded, Hank's solution or sterile Phosphate-buffered saline (2–4 CC) was added to it, and the flask content was removed again to completely remove all the remaining medium from the flask. At the same time, 1CC of trypsin (GIBCO, Grand, Island New York, US) was added to the medium-free flask. The flask was kept in a stationary condition for 1 min. The flask contents were then poured into the sterile falcon tube and were centrifuged at 1500 rpm for 10 min. Subsequently, the tube was placed under the hood at rest. The liquid was later removed from the tube, and the culture medium was re-added to it, and its amount was set at 1 mL. Under these circumstances, the amount of suspension cells was counted using an optical microscope (Zeiss, UK) and Neubauer slide (Sigma Aldrich, USA). Once the number of cells reached 500 thousand cells per mL, the cells were entered into four plates. A volume of 100 mL cell alongside 200 mL medium was added into each plate. The plates were later incubated for 2 h and were examined under an inverted microscope to determine the extent of cell adhesion to the bottom of the plate. Thereafter, the supernatant was removed and the culture medium was added to it. At this stage, the tested samples were exposed to cells and four plates were prepared for testing.

- Plate 1: Conventional CH labeled with tetracycline with the concentration of 1 g/cc
- Plate 2: Conventional CH labeled with tetracycline with the concentration of 0.1 g/cc
- Plate 3: NCH labeled with tetracycline with the concentration of 1 g/cc
- Plate 4: NCH labeled with tetracycline with the concentration of 0.1 g/cc.

After 72 h of material effect on the medium, extra materials were removed by normal saline, and the absorption of the materials by the cells was evaluated under a fluorescence microscope. Sixteen areas were randomly selected from each plate, and the percentage of change in the fluorescence color that represents the absorption rate was measured. The percentage of fluorescence color was measured with "Lum" parameter of colors in Paint software [Figure 2].

Kruskal–Wallis analysis was used for overall comparisons. A series of Mann–Whitney U tests were used for pair-wise comparisons ($P < 0.05$).

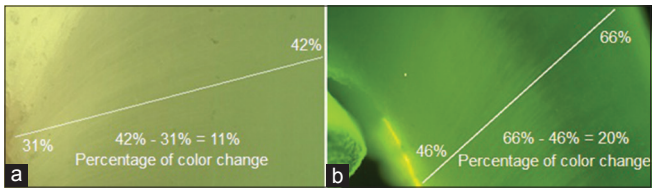

Figure 1: Measurement of the percentage of color change in the fluorescence in both ends of the spectrum (both white line ends) in the dentinal tubules by fluorescence microscopy: (A: conventional calcium hydroxide, B: nanoparticle calcium hydroxide).

RESULTS

Dentinal tubules penetration

Results showed that, in all three regions of the root, the percentage of fluorescence color change in NCH was more than the one with the conventional CH and the difference was significant ($P < 0.001$). Among the regions of the root for both materials, the lowest

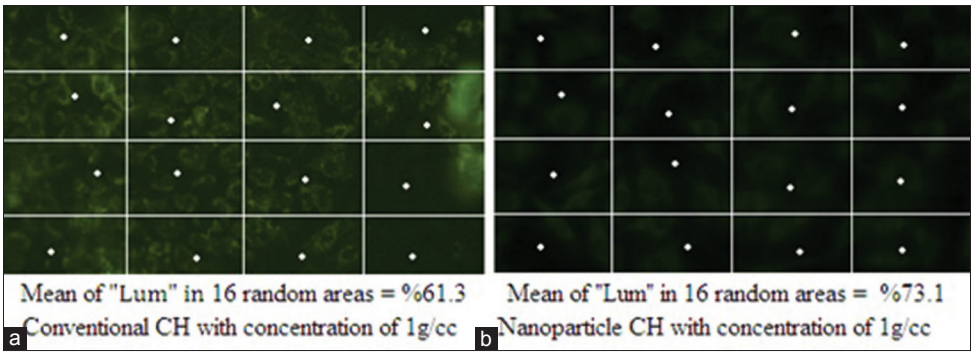

Figure 2: Measuring the percentage difference of the fluorescence color change in 16 random areas was due to the absorption of materials by L929 cells using fluorescence microscope: (A: conventional calcium hydroxide B: nonoparticle calcium hydroxide).

and highest percentage differences were related to the conventional CH in the cervical third (25% ± 1.6%) and NCH in the apical third (44% ± 1.2%) regions, respectively [Table 1].

L929 fibroblast cells penetration

Results showed that the percentage of fluorescence color change in drug with the concentration of 1 g/cc was more than the one with the concentration of 0.1 g/cc and the difference was significant ($P < 0.001$). The lowest and highest percentage of fluorescence color changes was obtained using conventional CH with the concentration of 0.1 g/cc (46% ± 2.6%) and NCH with the concentration of 1 g/cc (71% ± 1.9%), respectively [Table 2].

Table 1: Mean values of the percentage difference of the fluorescence color change after one week (mean±standard deviation)

Drugs	Root area		
	Cervical third	Middle third	Apical third
Conventional CH (Percent)	25±1.6	27±2.4	32±2.1
NCH (Percent)	35±2.6	43±2.9	44±1.2

NCH: Nanoparticle calcium hydroxide; CH: Calcium hydroxide

Table 2: Mean values of the percentage difference of the fluorescence color change after 72 h (mean±standard deviation)

Drugs	Concentration	
	0.1 g/cc	1 g/cc
Conventional CH (Percent)	46±2.6	62±4.4
NCH (Percent)	58±6.4	71±1.9

NCH: Nanoparticle calcium hydroxide; CH: Calcium hydroxide

DISCUSSION

In this study, the initial speculation was that nanosizing of drug particles and the production of drug in the form of nanoparticles is associated with the following advantages: drug gradually exert its effect in the root channel, longer shelf life, penetration into the dentin through the dentinal tubules, and high efficiency in the elimination of microorganisms. In recent years, similar researches which were carried out to evaluate the permeability of nanoparticles attempted to have proved their potential for greater penetration compared with the conventional drug.[19] Nevertheless, no study has investigated the permeability of NCH yet; however, many studies have investigated the permeability feature of CH and other newly-made drugs against oral pathogens like Foster *et al*. evaluated CH diffusion through radicular dentin with pH measurement[20] and Al-Hezaimi K compared antibacterial effect of two mineral trioxide aggregate (MTA) preparations against *E. faecalis* and Streptococcus sanguis *in vitro*[21] and Azarsina *et al*. evaluated antibacterial properties of composite resin containing nanosilver against *Streptococcus mutans* and *Lactobacillus*.[22] Each of these nanoparticles is unique considering characteristics such as size, shape, and concentration of nanoparticles used as well as surfactants and stabilizers composition, and these characteristics are also effective on their properties.[23] For instance, antimicrobial effects of silver nanoparticles have been considered and analyzed in recent years. Most of these researches concluded that silver nanoparticles, at very low concentrations, inhibited the growth of microorganisms and attributed this stronger antimicrobial property to the increase in the surface to volume ratio and higher drug penetration and persistence in the relevant environment.[24] Since conventional CH drug is the most commonly used drug among dentists within the canal; many studies have examined it in various compositions and properties.

Obviously, the methodology of these researches was different in many cases, and perhaps, this difference may lead to a variety of results obtained.[25] Fluorescence microscope is employed to determine the depth of penetration of material into the tooth structure and their absorption by different cells.[26]

There are many methods that are used to evaluate the permeability of CH such as scanning electron microscope (SEM), dental tubular leakage, and pH measurement. This high-resolution fluorescence microscope has many special advantages over other methods such as investigates samples without killing them, more precision, and lower time. Investigating samples by fluorescence microscope is associated with some disadvantages, including the surface which is evaluated in a two-dimensional manner. On the other hand, preparation of living samples before their investigation by the fluorescence microscope may cause artifact.[27] Jain A (2016) compared the root-end sealing ability of four different retrograde filling materials in teeth with root apices resected at different angles. The aim of this study was to compare the sealing ability of four root-end filling materials MTA, portland cement, intermediate restorative material, and resin-modified glass-ionomer cement in teeth with root apices resected at 0 and 45 angle using dye penetration method under fluorescent microscope.[28] The method was as same as our method.

In this study, the penetration depth of materials into the dentinal tubules and absorption by L929 cells was evaluated employing fluorescence microscope. Penetration and absorption values obtained from the tested drugs reveal that in all the regions of root and concentrations, NCH drug is penetrated and absorbed more than the conventional CH drugs. In fact, these results are consistent with SEM results, which demonstrate that nanoparticles drug can spread more easily within the dentinal tubules and passes through the cell membrane more easily. Therefore, it seems that nanosizing of drug particles and increasing the surface to volume ratio helps small amounts of the drug to have the same efficacy as the conventional drug. The only interesting point of our emphasis was on labeling drugs utilizing tetracycline to identify them by the fluorescence microscope in this test, which may lead to some changes in the shape, dimensions, and properties of the materials. Nevertheless, despite the importance of such experimental studies in evaluating the performance of dentinal materials, it should be noted that there are several constraints facing generalization of the results of these studies in clinical settings and patents' mouth since it is easy to use materials and conduct clinical processes in the laboratory environment. It is also easy to control the confounding variables, while there many confounding variables in the oral environment that are very difficult to control.[29] On the other hand, the complex anatomy of the root canal system has also made it impossible to completely debride it so that even after cleaning, shaping, and cleaning of canal using different mechanical and pharmacological agents; it is not possible to completely eliminate bacteria.[30] Although it is impossible to completely generalize results of laboratory methods in the clinical setting, researchers have considered their application in comparing different drug regimens and screening materials and practical techniques. Therefore, it seems necessary to conduct further research utilizing other laboratory tests or in clinical trials to evaluate the biocompatibility properties of materials on the root canal to reach firm conclusions regarding the efficacy of biocompatibility of materials and various drugs used in the root canal. The nanosizing of the drug particle in many materials leads to an increase in the impact and reduction in the dose of the drug, which is considered a very important advantage due to the reduction of adverse effects of such a material. In addition, more clinical studies should be carried out on the NCH.

CONCLUSION

The nanoparticle drug compared with the conventional drug has a more penetration depth in all regions of the root of the dentinal tubules. Furthermore, the nanoparticle drug demonstrated more penetration depth in L929 cells in all concentrations compared to the conventional drug. This high penetration possibly will increase antibacterial effects than the conventional drug.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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