博 士 論 文

Effects of different calcium hydroxide pastes on dentine resorption of osteoclasts in vitro

(異なる水酸化カルシウム製剤が in vitro における破骨細胞による象牙質吸収に与える影響)

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INTRODUCTION

1. Background

Dental trauma, such as avulsion and luxation, often involves damage to teeth and the supporting tissues. This can lead to pulp necrosis, root canal infection and external infection-related resorption (EIR)[1]. Microorganisms from infected root canals can migrate through dentinal tubules to the external resorption area, causing the persistence of EIR[2]. Eliminating microorganisms in the root canal system is essential to prevent and prohibit EIR. Calcium hydroxide medicament is recommended as the intracanal medicaments for tooth with a necrotic pulp associated with a luxation injury[3].

Calcium hydroxide in root canals not only exhibits antibacterial and endotoxin-neutralizing properties but also positively influences the resorption site's local environment by neutralizing acids and collagenases released by resorptive cells[4]. The alkaline pH at the resorption site stimulates alkaline phosphatase activity, promoting root surface repair[5]. In the root canal, calcium hydroxide paste effectively eliminates bacteria through direct contact[6]. However, microorganisms in the complex structures of dentinal tubules present a significant challenge. There is no direct way to physically clean these areas, so improving the penetration of intracanal medicine into these tiny structures is a practical solution. The diffusion of calcium hydroxide through dentinal tubules to the resorption area can significantly enhance the treatment efficacy for EIR. Therefore, the penetrability of calcium hydroxide pastes as an intracanal medicament has always been a focus for researchers.

Factors Affecting Penetration

1) Vehicle

Calcium hydroxide, a white, odorless powder, requires a vehicle to function effectively as an intracanal medicament. The choice of vehicle in calcium hydroxide paste is crucial, as it influences the paste's dissolution, ion release. Initially, distilled water was used as the vehicle for calcium hydroxide pastes; however, various formulations with different vehicle types have since emerged. Vehicles are typically classified based on their water affinity into aqueous, viscous, and oily types[7]. Aqueous vehicles, such as distilled water, and viscous vehicles, such as polyethylene glycol, are hydrophilic vehicle. In contrast, oily vehicles are hydrophobic vehicle.

Previous studies have reported varying results regarding the effectiveness of different

vehicles in promoting ionic dissociation and diffusion. For instance, some studies report that propylene glycol, a viscous vehicle, improves penetrability more effectively than distilled water[8], whereas others indicate that higher concentrations of viscous agents, such as glycerin, reduce ion release[9]. Oily vehicles, being water-insoluble, show low solubility and diffusion in tissues, allowing for extended retention within the root canal[10].

2) Additional Components

Calcium hydroxide has excellent antibacterial properties but is not universally effective against all bacteria in the root canal system[11]. To improve its efficacy, antibacterial substances are often added. For example, camphorated p-monochlorophenol (CMCP) has a broad antibacterial spectrum and acts more quickly than calcium hydroxide alone[12]. Combining calcium hydroxide with agents like iodoform or silicone oil enhances its antibacterial effect without necessarily affecting penetration. However, some additives, such as chitosan, may decrease penetration depth due to their gel-like properties, which limit diffusion into dentinal tubules[13].

3) Ultrasonic Activation

Ultrasonic activation is another factor influencing penetration depth. Studies indicate that ultrasonic activation can enhance ionization, enabling calcium hydroxide particles to penetrate deeper into the dentinal tubules[14]. This method improves antibacterial effectiveness and promotes deeper diffusion of calcium hydroxide. However, the impact of ultrasonic activation on penetration is not entirely consistent. Some studies report limited benefit from ultrasonic mixing compared to sonic mixing or other techniques, possibly due to differences in experimental conditions, ultrasonic equipment, or paste composition[8].

4) Nanoparticles

Recent research shows that nanoparticles may provide enhanced penetration due to their smaller size, allowing deeper access into the microstructure of dentinal tubules. Commercially available calcium hydroxide pastes that contain smaller, smoother particles have demonstrated better permeability compared to those with larger particle sizes[15]. Calcium hydroxide nanoparticles achieve deeper penetration and maintain their antibacterial effectiveness longer, making them a promising option for intracanal medications. Polyethylene glycol, another viscous carrier, has been shown to enhance the deeper penetration of fine calcium hydroxide particles into dentinal tubules by reducing particle size[16].

Methods for Evaluating Penetration

1) pH Measurement

Calcium hydroxide pastes release calcium ions and hydroxide ions, which have antibacterial properties mediated by various mechanisms, including microbial cell membrane damage, DNA damage, inhibition of DNA replication, suppression of enzyme activity, and disruption of cellular metabolism[17]. Additionally, hydroxide ions diffuse through dentinal tubules to the root surface, increasing the local pH. When pH exceeds 7.4, osteoclast activity is inhibited, reducing root resorption[1,18,19].

Researchers often use pH changes to evaluate calcium hydroxide penetration by comparing pH values before and after paste application. Tronstad's study showed that reimplanted and non-reimplanted teeth treated with calcium hydroxide had dentin pH levels of 8.0 to 11.1 near the pulp and 7.4 to 9.6 in peripheral dentin[20]. This indicates that calcium hydroxide in the root canal positively affects the local pH in resorption areas. Other studies also confirmed significant pH changes in dentin or external solutions after placing calcium hydroxide in the root canal[21,22,23]. Lots of factors influencing hydroxide ion diffusion have been studied, including different carrier properties[21,24,25], the addition of other components[26,27,11], ultrasonic activation[14,28], differences in measurement sites within the root[22], and comparisons with other materials like MTA[29,30,31].

2) Ion Concentration Analysis

Another indirect method for evaluating penetration is measuring changes in calcium ion concentration, which reflects the paste's diffusion ability. Like pH measurement, this approach has similar limitations, as variations in sample types and measurement locations can lead to inconsistent data[32]. Studies show that calcium ions can diffuse to the outer root surfaces, suggesting effective penetration, but more precise and standardized methods are needed to confirm these findings consistently.

3) Scanning Electron Microscopy (SEM)

SEM offers detailed images of dentinal tubules by scanning the sample surface with a high-energy electron beam. This technique reveals the surface morphology, composition, and crystalline structure of the tubules, allowing direct visualization of calcium hydroxide particles within them[33]. However, SEM requires complex sample preparation, including dehydration and sputter coating in a high-vacuum environment, which can disrupt or move particles, potentially affecting the accuracy of penetration depth measurements. Although SEM provides high-resolution images, it only captures small areas at a time, making large-scale data collection challenging. Additionally, the sample preparation process may alter the physical integrity of calcium hydroxide

particles, complicating penetration assessment[34].

2. Research Objectives

To conduct a comparative study on the effects of hydrophilic and hydrophobic calcium hydroxide medicaments in treating EIR, this research established an in vitro model to observe the penetration capability of these intracanal medicaments through dentinal tubules and their impact on osteoclast activity cultured on the root surface. This study is divided into two parts, each of which will be described in detail in the paper.

PART I

The Penetrability of Hydrophilic and Hydrophobic Calcium Hydroxide Pastes in Dentinal Tubules

1. Introduction

Vehicle in calcium hydroxide paste influences the paste's dissolution, ion release. The dissociated ions diffuse through the dentinal tubules, which can be explained by dentinal penetrability governed by tubular anatomy, density, diameter, and length.
The penetrability of calcium hydroxide medicaments plays a crucial role in the treatment of EIR and the success of pulp therapy. Therefore, the penetrability has consistently been a focus of research.

Extensive research has explored the factors affecting the penetrability of calcium hydroxide medicaments, focusing on the type of vehicle, additional components, and agitation methods and so on. Among these, the vehicle is a critical factor. Some studies support the conclusion that aqueous vehicles enable the fastest initial ion dissociation and diffusion, while viscous carriers support a slower, sustained ion release[25]. These insights are essential for the informed selection of vehicles in clinical practice, based on the specific therapeutic requirements for calcium hydroxide diffusion and retention.

Calcipex® II (Nippon Shika Yakuhin, Tokyo, Japan) and Vitapex® (Neo Dental Chemical Products, Tokyo, Japan) are calcium hydroxide-based medications commonly used in clinical practice in Japan, Calcipex® II as a hydrophilic paste and Vitapex® as a hydrophobic paste. Currently, there is an absence of comparative research on the penetration ability of the two commercial products. Therefore, this study aims to conduct a comparative analysis of these two products and employs pH measurement as a method to evaluate the penetration capability of both hydrophilic and hydrophobic calcium hydroxide pastes within dentinal tubules. The primary objective is to establish a new in vitro model of EIR that closely simulates the clinical conditions under which calcium hydroxide-based medicaments are utilized for the treatment of external root resorption.

2. Materials and Methods

2.1 Root Samples Preparation

A total of 18 extracted human permanent teeth with a single root and single canal had been stored in the laboratory. Teeth that had undergone endodontic treatment, displayed resorption, cracks, or had incomplete root formation were excluded from the study. After thorough cleaning and disinfection, the selected teeth were stored in normal saline at room temperature until use.

The crowns of the teeth were removed using a Maruto cutter (MARUTO, Tokyo, Japan). The root canals were instrumented to a #70 K-file (ISO), maintaining a working distance of 0.5 mm from the anatomical apex. To create a flat plane parallel to the tooth axis, part of the cementum on one side of each root surface was removed, ensuring a consistent thickness of 1 mm from this plane to the root canal.

For irrigation of the prepared plane and root canals, a sequence of 14% EDTA solution, 10% NaOCl solution, and sterile physiological saline was applied using an ultrasonic device (ENAC OE-W10, Osada, Tokyo, Japan). Each solution was applied three times for 15 seconds each, followed by drying with paper points.

2.2 Experimental Models Building and pH Measurement

The roots were divided into three groups according to the intracanal medicament applied: Calcipex II group (n=6)**:** The root canals were filled with Calcipex® II (Nippon Shika Yakuhin, Tokyo, Japan). Vitapex group (n=6)**:** The root canals were filled with Vitapex® (Neo Dental Chemical Products, Tokyo, Japan). Control group (n=6)**:** The root canals were left unfilled. All roots were sealed at root apex and orifice of root canal with Super-Bond™ Dental Adhesive (SUN MEDICAL CO., LTD, Shiga, Japan).

A 3 mm × 4 mm hole was drilled in each of the two central wells of a 12-well plate, and the prepared roots were attached to the bottom of the plate with the prepared plane aligned directly under the 3 mm × 4 mm holes. Using two 12-well plates, 4.5 ml of sterile physiological saline solution was added to the sample wells in the upper layer, while the corresponding wells in the lower layer were filled with sterile physiological saline solution to fully submerge the tooth roots (Fig. 1). pH measurements were recorded on days 0, 3, 5, and 7 by pH Meter (LAQUA, HORIBA, Kyoto, Japan). Data were statistically analyzed with one-way ANOVA and Brown-Forsythe test.

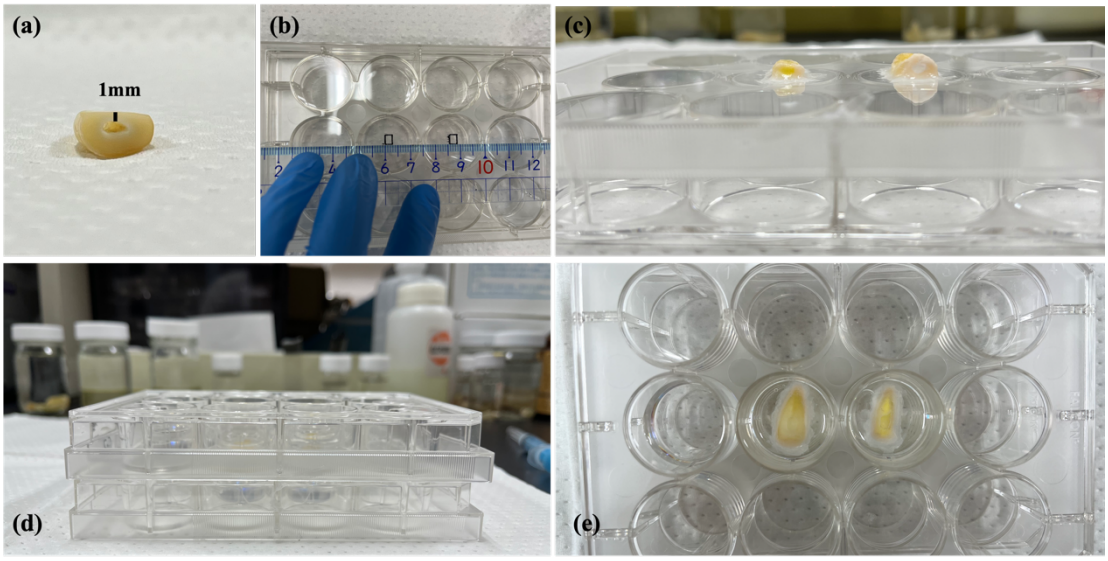


Fig. 1 Experimental model. **(a):** Remove part of cementum and dentine on one side of root to make a flat plane and ensure a thickness of 1 mm from this plane to the root canal. **(b):** A 3×4 mm^2 hole was made in each of the two central of a 12-well plate. **(c):** Prepared roots were fixed to the bottom of the plate. **(d):** Using two 12-well plates, the plate with teeth is placed on the top. **(e):** Ensure that the plane directly beneath the hole is positioned to allow direct contact with the solution.

3. RESULTS

The pH values of the Calcipex II and Vitapex groups exhibited a steady increase over the 7-day observation period, with a noticeable rise during the early 3 days (Fig. 2). By day 3, both experimental groups had reached an alkaline pH level, indicating that the medicaments produced an alkaline environment early in the experiment period. In contrast, the control group maintained a stable pH value throughout 7 days.

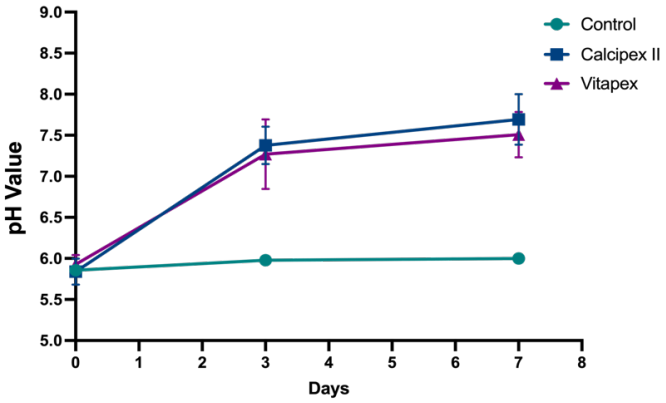
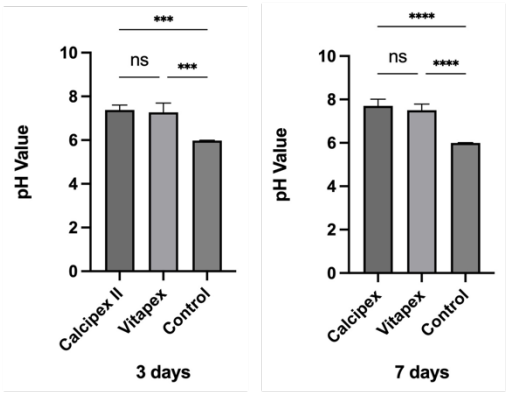


Fig. 2 The variation in mean pH values. pH values measured at different time intervals (0 days, 3 days, and 7 days). It shows a noticeable rise in the early 3 days and in the third day, Calcipex II group and Vitapex group had reached an alkaline pH level.

Comparative analysis at days 3 and 7 (Fig. 3a) showed significant differences in pH levels between the Calcipex II and Vitapex groups compared to the control group, with both medicated groups displaying higher pH values. However, there were no significant differences between the Calcipex II and Vitapex groups, indicating similar efficacy in pH elevation. The table (Fig. 3b) further illustrate these differences in mean pH values across the three groups at 0, 3, and 7 days.



(a)

Follow-up Periods	Groups	Mean pH
0 day	Calcipex II	5.840
	Vitapex	5.927
	Contro	5.856
3 days	Calcipex II	7.378
	Vitapex	7.269
	Control	5.979
7 days	Calcipex II	7.693
	Vitapex	7.507
	Control	5.999

(b)

Fig. 3 Comparison of the mean pH. **(a)** Comparison of the mean pH values among the three groups at 3 days and 7 days (***$p<0.001$; ****$p<0.0001$; ns: $p>0.05$); **(b)** The mean pH value of three groups at 0, 3 days, 7 days

4. Discussion

4.1 Penetrability of Hydrophilic and Hydrophobic Calcium Hydroxide Pastes

Researchers have suggested that the penetrability of calcium hydroxide pastes varies depending on the type of vehicle used. This study evaluated the penetrability of hydrophilic and hydrophobic calcium hydroxide pastes.

The pH values of the hydrophilic Calcipex® II paste and the hydrophobic Vitapex® paste showed a continuous increase over the 7-day period. This increase is attributed to the dissolution of the calcium hydroxide medicaments within the root canal, with hydroxide ions penetrate through the dentinal tubules to reach the root surface, thereby diffusing into the sample solution surrounding the root and raising the pH. The results suggest that both the hydrophilic Calcipex® II paste and the hydrophobic Vitapex® paste exhibit excellent penetrability. This result is consistent with the findings of Tronstad's study showed that teeth treated with calcium hydroxide had dentin pH levels of 8.0 to 11.1 near the pulp and 7.4 to 9.6 in peripheral dentin[20].

The pH value increased rapidly during the first three days, with both groups exceeding a pH of 7 by day 3, indicating the formation of an alkaline environment within the hole. Over the following four days, although the pH continued to rise, the increase was much slower. This suggests that both types of paste exhibit a relatively fast penetration rate, enabling the early establishment of an alkaline microenvironment conducive to inhibiting root resorption.

However, the results indicated that hydrophilic and hydrophobic vehicles did not exhibit a significant difference in the penetrability of calcium hydroxide medicaments in the in vitro model. In this study, the relatively large probe size of the pH meter and the limited volume of the 12-well plate may have compromised measurement accuracy and reduced the statistical power to detect significant differences. Although a sample solution volume of 4.5 ml was selected after repeated preliminary tests, these limitations might still have influenced the outcomes.

4.2 Analysis of Experimental Model

Previous studies mentioned above, which evaluated penetrability of hydrophilic paste and hydrophobic paste using pH measurements, have not reached a consistent conclusion. Lack of standardized research model is a possible reason for this discrepancy.

A variety of samples, including human teeth, animal teeth, and synthetic plastic teeth, are used across studies. While human teeth provide the highest clinical relevance, they pose challenges in achieving standardization due to the complex anatomy of the root canal system, which remains difficult to control[35]. In contrast, artificial teeth offer consistency and repeatability but fail to replicate the complexities of natural root canals. Previous studies differ in the selection of pH measurement models. Some put the root into a container, some fix the root in wax. pH measurement sites ranged from the inner or outer dentin layers to the solutions surrounding the roots. Additionally, variations in the types and volumes of these solutions further complicate the comparison of results. Differences in pH Measurement Instruments: Different materials and solutions require specific pH meters. The use of incompatible meters can result in unstable readings and significant measurement errors[36]. Some influence come from extraneous materials, such as the intrinsic pH of the tooth and residual irrigates within the root canal may affect pH readings, introducing potential confounders into the results[20]. To overcome these limitations, it is recommended to improve experimental protocols and use advanced technologies. Combining pH measurement with other methods could offer a more complete evaluation of medicament penetration, instead of depending only on pH changes[37,38].

Due to the lack of standardized experimental models for reference, this study attempts to develop a new model designed to more closely replicate clinical conditions. The

model design must fulfill the following requirements: 1. The calcium hydroxide medicament within the root canal should be capable of permeating through the dentinal tubules to reach the root surface. 2. Osteoclasts should be able to be cultured on the root surface, allowing observation of the effect of calcium hydroxide, once it has penetrated to the root surface, on the activity of the osteoclasts cultured there. Thus, two layers of 12-well plates were selected for the experiment, allowing hydroxide ions to reach the root surface and create a localized alkaline microenvironment in the hole. The hole simulates the local resorption microenvironment and replicate the clinical pathway of intracanal medicaments, allowing pH measurements to more accurately analyze localized effects of the medicaments.

5. Conclusions

Both the hydrophilic Calcipex® II paste and the hydrophobic Vitapex® paste exhibit excellent penetrability, especially in the first three days. In this part, the effectiveness of this designed model has been validated. Calcium hydroxide medicaments placed within the root canal can diffuse into the solution in the upper layer, creating an alkaline microenvironment suitable for observing osteoclast activity in the second step of the study.

Part II

Comparing the differences on effecting the activity of osteoclasts between hydrophilic and hydrophobic Calcium Hydroxide Pastes

1. Introduction

Current quantitative studies of osteoclastic bone resorption or teeth resorption have generally been based on experiments conducted in in vitro cell culture[39,40] or in vivo sectioned tissues[41,42,43]. Most previous studies have used dentin slices to culture and observe osteoclastic resorption activity on dentin. This study represents the first attempt to culture cells on an intact root surface, presenting a novel approach to investigating osteoclast behavior in a more physiologically relevant in vitro model.

There are also some advanced technologies that can directly observe the activity of cells inside animals, such as intravital imaging by two-photon excitation microscopy (TPEM) has been widely used to visualize cell functions. However, due to the current limitations of probe technology, such as small molecular probes (SMPs), commonly used for cell imaging, cannot be simply applied to intravital imaging because of the challenge of delivering them into target tissues, as well as their undesirable physicochemical properties for TPEM imaging[44]. Considering TPEM experiments are complex to operate and incur high costs in this study. Osteoclast activity had been evaluated using conventional analyses such as TRAP staining of osteoclasts or pit-formation assays [45,46].

This research established an in vitro model that osteoclast can be cultured on the root surface of a whole root which filled with calcium hydroxide medicaments. This model partly reproduced the intracanal medicaments how to penetrate dentinal tubules to affect the activity of osteoclasts.

2. Materials and Methods

2.1 Cell Culture

Osteoclasts precursor cells were purchased from COSMO BIO CO., LTD (Rat Osteoclast, Code No. OSC12C), The methods for cell revival and cultivation were carried out according to the protocols provided by the company. Cells were seeded into 12-well-

plates at a cell density of 4 x 10^5 cells/ml. The 12-well-plates were prepared as part I and were used following rigorous disinfection procedures. The cells cultured in culture media containing RANKL, MCSF. Then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 7 days, replacing the culture medium every two days.

2.2 Tartrate Resistant Acid Phosphatase (TRAP) Staining

At the end of cultures, the culture medium was removed. The cells were washed by PBS, fixed in glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 12h at 4°C. Stain cells with TRAP staining Kit (Catalog No. AK04F, COSMO BIO CO. LTD), for 60 min at 37°C. Plates were washed three times with distilled water and left to dry.

2.3 Observation and Counting of TRAP-positive Osteoclasts

The cells were observed under a stereoscopic microscope (SMZ-10, NIKON, Tokyo, Japan). Osteoclast morphology on the root surface within the 3×4 mm² hole, as well as on the surrounding well surface, were observed at 1x, 2x, and 4x magnifications. At 4x magnification, in each sample well, images were captured at the top, bottom, left, and right of the hole, along with two images focused on the dentin within the hole. The same setting of color balance, brightness, and contrast was adjusted for each image. TRAP-positive cells over 100μm were counted and recorded. The density of TRAP-positive cells, which indicates the frequency of the appearance of TRAP-positive cells, was computed from the number of TRAP-positive cells and the area of the root surface or plate surface of each image (cell density=numbers of TRAP+ cells/area of image).

2.4 Observation and Counting of Root Resorption Pits

The root samples were removed from the 12-well plate. To facilitate observation under scanning electron microscope (SEM), a portion of the non-observed root tissue was abraded. The root samples were incubated in 5.0% sodium hypo chloride in an ultrasonic bath for 5 minutes to remove cultured cells and cribs from the root surfaces. They were then dehydrated through a graded series of ethanol and critical point dried with liquid CO_2. The dried slices were coated with platinum and palladium in an ion coater (HITACHI E-1030, HITACHI, Tokyo, Japan). Then observed under SEM (SEM; HITACHI S-4000, HITACHI, Tokyo, Japan), at an accelerated voltage of 10 kV.

The observation area (3×4 mm²) was divided into 12 fields of view. Each sample was observed and photographed under magnifications of 30×, 50×, 100×, 200×, and 400×. To minimize interference from the morphology of dentinal tubule openings and considering the inclusion criteria for TRAP-positive cells greater than 100 μm in the previous experiment, resorption pits larger than 50 μm were included in the count at

50× magnification. The resorption pits observed at 50× magnification were further examined and confirmed for their morphology at 100× and 200× magnification before being counted.

2.5 Data analysis

Data are presented as mean ± standard deviation (SD). The densities of TRAP-positive cells inside and outside the hole in each group were compared using F-test and Student's t-test. The analysis of one-way ANOVA and Brown-Forsythe test were used for the comparison of means from the three groups. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA), $p<0.05$ was considered as significant.

3. Results

3.1 Numbers of TRAP-positive Osteoclasts

The results indicate that the growth and differentiation status of osteoclasts varied across groups. In this study, TRAP-positive cells with diameters greater than 100 μm were included in the cell count. Osteoclasts containing 3-4 nuclei typically measuring 60-80 μm. Given the low magnification used in this study, we selected TRAP-positive cells with diameters greater than 100 μm to enhance the accuracy of the cell count. Images showed that TRAP-positive cells in the control group appeared larger and were more intensely stained. The number of TRAP-positive cells in the control group was significantly higher than in the other two experimental groups ($p<0.01$). Additionally, the Calcipex II group had significantly fewer TRAP-positive cells than the Vitapex group ($p<0.001$) (Fig. 4).

Within-group comparisons were conducted to analyze the number of TRAP-positive cells inside and outside the hole. Cell density, defined as the number of TRAP-positive cells per unit area, was used as the evaluation metric. The formula for cell density is cell density = number of TRAP-positive cells / area of the image. At 4× magnification, all images had the same area, measured at 8 mm^2. In all groups, the number of TRAP-positive cells attached to the root surface inside the hole was lower than the number attached to the plate surface surrounding the hole. Both the Calcipex II group and the Vitapex group exhibited significantly greater differences ($p<0.01$) compared to the control group ($p<0.05$) (Fig.5).

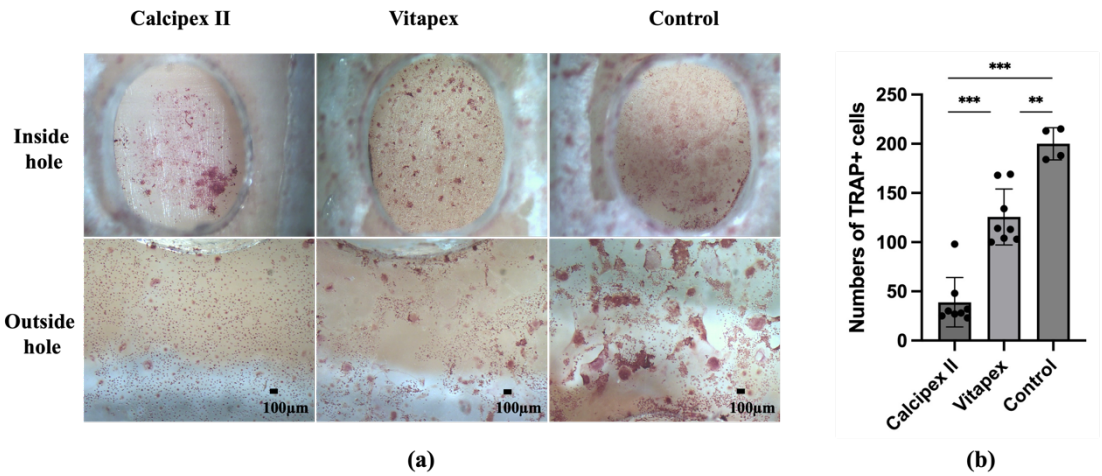


Fig. 4 Different effect of hydrophilic and hydrophobic calcium hydroxide pastes on the numbers of TRAP-positive osteoclasts. **(a)** Representative images of TRAP-positive cells of each group. The TRAP-positive cells in the control group appear larger in size and more intensely stained. **(b)** Quantification of TRAP-positive osteoclasts. TRAP-positive cells larger than 100 μm were included in the count. The numbers of TRAP-positive cells in the control group were significantly higher than in the other two groups. The calcipex II group had significantly fewer TRAP-positive cells than the vitapex group. (**p<0.01; ***p<0.001)

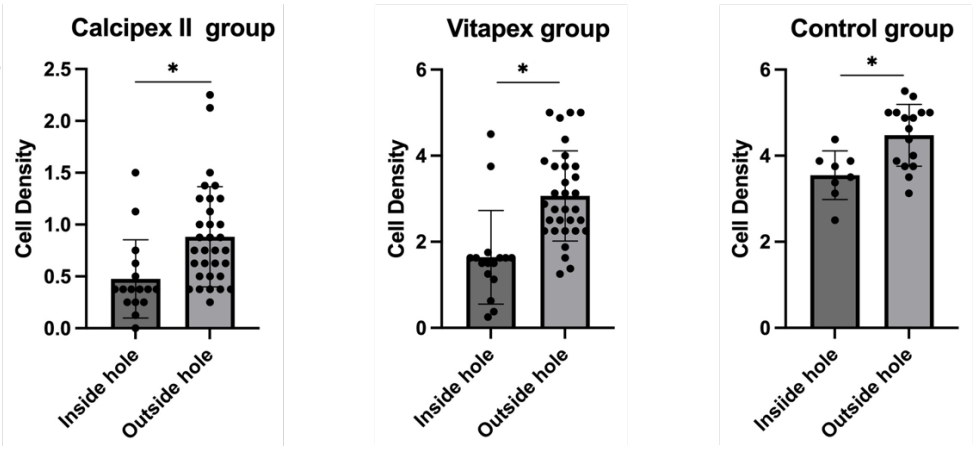


Fig. 5 Comparisons between the numbers of TRAP-positive osteoclasts inside and outside the hole of each group. Cell density = numbers of TRAP-positive cells/area of image (at 4x magnification, the area of all the images is same, area of image is 8mm^2). In all groups, the numbers of TRAP-positive cells attached to the root surface inside the hole was lower than that to the plate surface surrounding the hole. Both the Calcipex II group and the Vitapex group showed significantly greater differences (**p<0.01) compared to the control group (*p<0.05).

3.2 Numbers of Resorption Pits

Samples from the three groups were observed and counted under the SEM at different magnifications. Some noteworthy observations were made. Even after treatment with sodium hypochlorite solution and ultrasonic cleaning, a few larger osteoclasts remained on the root surface (Fig. 6). Resorption pits larger than 50 μm were measured. Among the three groups, the Calcipex II group showed the fewest resorption pits on the root surfaces. In the representative samples shown (Fig. 7a), TRAP-positive cell attachment areas were clearly observed on the Calcipex II root surfaces under stereoscopic microscope, but at the same area no significant resorption pits were seen under the SEM. In contrast, the control group exhibited many distinct pits visible under the SEM. Statistical analysis showed significant differences among the three groups. (*$p<0.05$; **$p<0.01$; ***$p<0.001$) (Fig. 7b).

The Pits/TRAP+ cells ratio represents the relationship between the number of counted TRAP-positive cells and the number of counted resorption pits. This ratio reflects the proportion of TRAP-positive cells with resorption activity. The Calcipex II group showed the lowest ratio, with a significant difference compared to the control group, indicating that the proportion of cells with resorption activity in the Calcipex II group was lower than in the control group. No significant differences were observed among the other groups (*$p<0.05$; ns: $p>0.05$). (Fig. 7c)

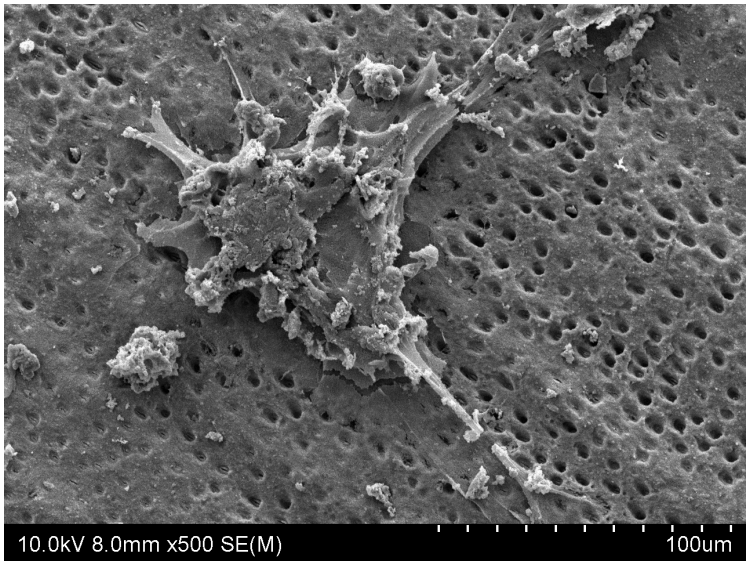


Fig. 6 Lager osteoclast observed under SEM. Some osteoclasts that were not completely removed by ultrasonic bath can still be seen on the dentin surface.

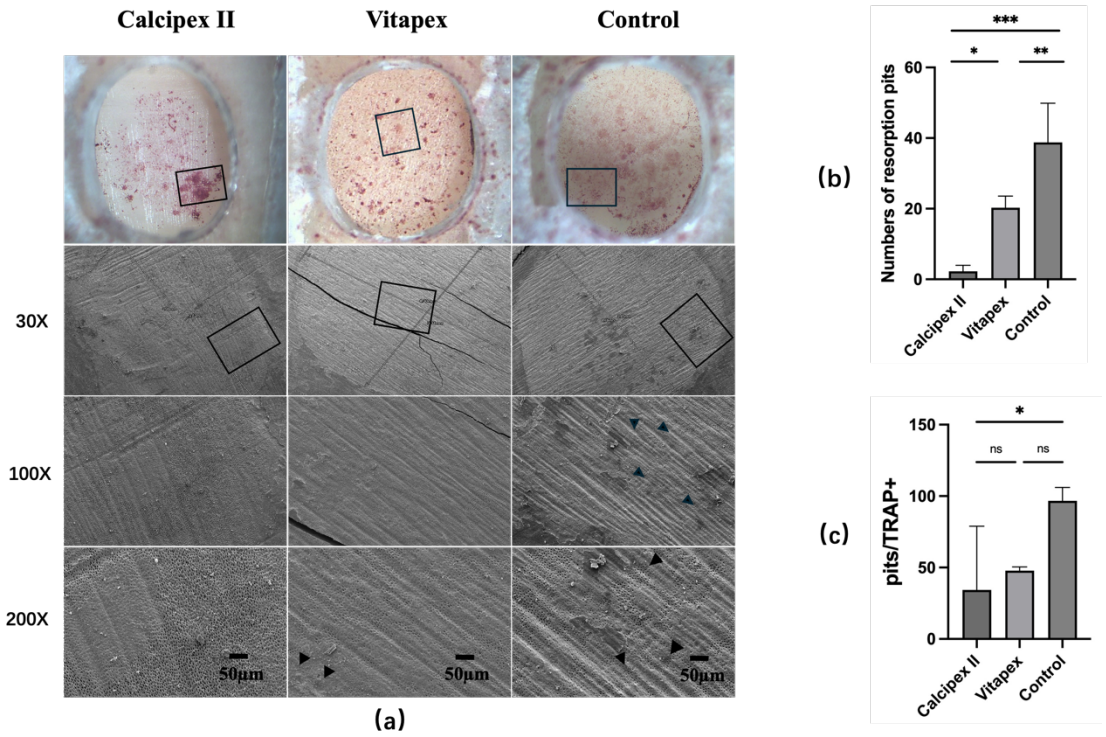


Fig. 7 Numbers of resorption pits and pits/TRAP+. **(a)** The observation area under the SEM corresponds to the size of the prepared holes. Images shown the resorption pits taken at 30x, 100x, and 200x magnifications. (arrow heads: resorption pits) **(b)** The number of resorption pits was compared among the three groups, revealing significant differences. **(c)** The ratio of the number of pits to the number of TRAP-positive cells reflects the proportion of TRAP-positive cells exhibiting resorption activity. A significant difference was observed in this ratio between the Calcipex II group and the control group, while no significant differences were noted among the other groups. (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ns: $p>0.05$)

4. Discussion

The type of vehicle significantly influences the decomposition and ion diffusion capacity of calcium hydroxide pastes, which is a critical factor affecting their clinical efficacy. The diffusion of calcium ions and hydroxide ions into dentinal tubules is particularly important for the treatment of external root resorption. Calcipex® II and Vitapex® are two commonly used calcium hydroxide-based intracanal medicaments in clinical practice. Calcipex® II is a hydrophilic paste, while Vitapex® is hydrophobic. This study investigates the effects of these medicaments on osteoclastic activity at the root surface. The goal is to provide evidence-based guidance for clinical decision-making in the selection of intracanal medicaments.

In Part I of this study, we developed an in vitro model to simulate the placement of

intracanal medicaments and assessed the ability of Calcipex® II and Vitapex® to penetrate dentinal tubules by measuring pH changes. The results demonstrated that hydroxide ions from both medicaments diffused to the root surface within three days, establishing a localized alkaline microenvironment. Osteoclasts, the primary cells responsible for external root resorption, exhibit reduced activity under alkaline conditions but demonstrate enhanced resorptive activity in acidic environments[44]. The localized alkaline environment generated in Part I provides the necessary baseline condition for the subsequent investigations in Part II. Therefore, the model established in Part I proved effective. Building on this foundation, we further examined whether the two medicaments differ in their effects on osteoclastic activity.

4.1 Alkaline conditions inhibit osteoclast differentiation

A comparison of the density of TRAP-positive cells inside and outside the holes in each group demonstrated that the density inside the holes was significantly lower than that outside. This observation suggests that the microenvironment within the holes is more effective in inhibiting osteoclast growth and differentiation.

From the perspective of molecular diffusion, ions move along a concentration gradient from regions of higher concentration to regions of lower concentration. In this model, the confined geometry of the holes creates a localized area of high hydroxide ion concentration, as the diffusion of ions first into the hole from dentinal tubules and then diffusion into surrounding medium. Therefore, the difference in TRAP-positive cell density between the inside and outside of the hole may be attributed to the variation in hydroxide ion concentrations. The accumulation of hydroxide ions inside the hole elevates the pH of the local microenvironment, making it higher than in other areas of the well. Other research has shown that osteoclast activity is sensitive to pH changes, with a pH above 7.45 significantly reducing their resorptive function and differentiation capacity[44]. This study also suggests that alkaline conditions inhibit osteoclast differentiation.

4.2 Effects of Different Calcium Hydroxide Pastes on Osteoclast Differentiation

Osteoclast precursor cells were seeded onto the root surfaces filled with Calcipex® II paste and Vitapex® paste and cultured for 7 days. The cell growth and differentiation in the Calcipex II group and Vitapex group were significantly less pronounced compared to the control group. Especially, the Calcipex II group exhibited fewest 100+ μm TRAP-positive cells and smaller cell sizes. Although both medicaments effectively inhibited the maturation and differentiation of osteoclast precursor cells, the hydrophilic Calcipex® II paste demonstrated a stronger inhibitory effect compared to the hydrophobic Vitapex® paste.

The observed difference may be attributed to the higher short-term ion diffusion efficiency of the hydrophilic paste compared to the hydrophobic paste. Hydrophilic vehicles are water-soluble substances with a high degree of solubility. When the paste comes into direct contact with tissue fluids, Ca^{2+} and OH^- ions are rapidly released. In contrast, oily vehicles, being non-water-soluble substances, minimize the solubility and diffusion of the paste within the tissues, resulting in slower ion release and lower immediate effectiveness[7,47].

4.3 Effect on the Resorptive Activity of TRAP-Positive Osteoclasts

The Calcipex II group exhibited the lowest number of resorption pits among all groups, indicating that osteoclasts in this group had the least resorptive activity. In contrast, osteoclasts in the Vitapex group displayed higher resorptive activity compared to the Calcipex II group. The control group showed the highest resorptive activity.

According to some research, osteoclasts have evolved specialized lysosome-related organelles (LROs), which are acidic vesicles dedicated to the secretion of bone-resorbing proteins. These vesicles are therefore referred to as secretory lysosomes[48]. Prolonged exposure to high alkaline conditions may compromise proton pump efficiency, leading to elevated lysosomal pH and impaired digestive function. Sustained alkalinity could also reduce the activity or inactivate lysosomal enzymes, such as acid phosphatase and cathepsin, thereby disrupting bone resorption and metabolism[49]. The rapid ion release and short-term ability of hydrophilic paste to establish a strong alkaline microenvironment better than the hydrophobic paste. As a result, the Calcipex II group more effectively inhibited osteoclast activity, resulting in the formation of the fewest resorption pits.

To assessed whether the TRAP-positive osteoclasts observed in this study possessed the ability to resorb dentine, this study utilized the ratio of the number of pits to TRAP-positive cells (pits/TRAP+) as an indicator to reflect the proportion of TRAP-positive cells exhibiting resorptive activity. TRAP-positive osteoclasts with a diameter greater than 100 μm were included in the assessment. The pits/TRAP+ ratio in the Calcipex II group showed a significant difference compared to the control group. This indicates that, although larger osteoclasts differentiated in the Calcipex II group, Calcipex® II suppresses the resorptive activity of differentiated osteoclasts.

However, the result does not provide sufficient evidence to conclude an inhibitory effect of Vitapex® paste. When compared separately with the Calcipex II group and the control group, the pits/TRAP+ ratio in the Vitapex group did not show any statistically significant differences. Several factors might explain this observation: 1. High data variability: The standard deviation for the Vitapex group was small, in contrast the Calcipex II group are relatively large (Fig.6c), which could have reduced the sensitivity of the statistical analysis. 2. Weaker effect of Vitapex® : While Vitapex® may have some

inhibitory effect on osteoclastic resorptive activity, its impact appears less pronounced compared to Calcipex® II.

Although the differences between the Vitapex group and the other two groups did not reach statistical significance, the trend of a lower pits/TRAP+ ratio in the Vitapex group compared to the control group may hold biological significance. By combining the analysis of the number of resorption pits and the pits/TRAP+ ratio, it can be inferred that Vitapex® also exerts a certain degree of inhibitory effect on osteoclast activity, weaker than that of Calcipex® II.

4.4 Analysis of the Discrepancy in Penetrability Between Part I and Part II

It is worth discussing that this result contradicts the findings from Part I of this study regarding the differences in penetration efficiency between hydrophilic and hydrophobic pastes. In Part I, it was observed that both the hydrophilic Calcipex® II and the hydrophobic Vitapex® paste caused a significant increase in pH within the first three days, with no significant difference between the two groups. This indicated that the penetration efficiency of hydroxide ions was similar effective for both pastes. However, in Part II, the two pastes exhibited notable differences in their effects on osteoclast differentiation, which indicated that Calcipex II group have the better penetrability than Vitapex group. Regarding penetrability, the two parts reached different conclusions.

Two potential factors could explain this discrepancy. Second, the experimental conditions differed between pH measurement and cell culture. For pH measurement, 4.5 mL of saline solution was added to each well and remained unchanged for the entire 7-day period. In contrast, during osteoclast culture, 2 mL of culture medium was used per well and was replaced every two days. The lack of medium replacement during pH measurement could have led to ion saturation within the solution, which might have inhibited further ion diffusion from the dentine tubules. This may unclear the differences of penetrability between the two groups. This is supported by the observation that the pH increased rapidly during the first three days; however, in the following four days, the pH rise was significantly slower.

5. Conclusions

The hydrophilic Calcipex® II paste demonstrates superior short-term penetrability compared to the hydrophobic Vitapex® paste, effectively suppressing osteoclast resorptive activity within a short period. The hydrophilic Calcipex® II paste is typically recommended when fast ion diffusion is needed, such as the treatment of EIR. The hydrophobic Vitapex® paste is more suitable for treatments requiring long-term and

sustained effects.

This study preliminarily explored a new in vitro experimental model for investigating EIR. Although some meaningful results were obtained, the experimental methods still require further refinement. Future studies could incorporate additional experimental techniques to strengthen the evidence, such as using Confocal Laser Scanning Microscopy (CLSM) for direct visualization and analysis of penetrability.

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