ALKALINE PHOSPHATASE ACTIVITY ASSESSMENT OF TWO ENDODONTIC MATERIALS: A PRELIMINARY STUDY

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Original Article

S. Rajan[1], H. Awang[1], S. Devi[2], A.H. Pooi[3], H. Hassan[2]

[1] *Department of Children's Dentistry & Orthodontics, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia.*
Tel: 79674540
Email: rsadna@um.edu.my

[2] *Department of Medical Microbiology,*

[3] *Institute of Mathematical Science,*

Corresponding author: Sadna Rajan

ABSTRACT

Objective: An *in vitro* assessment of MG-63 human osteosarcoma cells' alkaline phosphatase (ALP) activity when in contact with calcium hydroxide powder (CH), paste (CHP) and grey mineral trioxide aggregate (MTA).

Methods: MG-63 cells were seeded to the three selected materials for durations of 0.25, 0.5, 1, 24, 48 and 72 hours. BCIP-NBT assay was used and ALP activity quantified using ELISA reader at 410 nm.

Results: The overall analysis for ALP activity indicated significant interaction between test materials and control (maintenance medium). Subsequently, the test materials were paired and analysed for initial (0.25, 0.5, 1 hour) and delayed response (24, 48 and 72 hours). During the initial response, CH exhibited an increased ALP activity compared to MTA. This interaction was not dependant on duration. The delayed response exhibited elevated ALP activity with CHP when compared to MTA and CH. The interaction of CHP was dependant on duration.

Conclusion: All three materials exhibited increased ALP activity.

Key words: MTA, calcium hydroxide, alkaline phosphatase, *in vitro* assessment, MG-63 cell line

INTRODUCTION

Endodontic treatment is greatly complicated in non-vital immature permanent teeth. Pulp necrosis may occur at any stage of root development. The tooth would have an open apex and root apex may be divergent, parallel-shaped or convergent (1). In any event, the treatment of choice is apexification, whereby an osteoinductive material is placed into the root canal to induce hard tissue barrier formation at the root apex.

There are two types of apexification, conventional and one visit type. In conventional apexification, an osteoinductive material is placed into the canal and replaced periodically until a hard tissue barrier is formed. This process may take between 13-67weeks (2). The long duration needed for treatment and repeated replacement of canal medicaments would have implications on strength of tooth (3). In addition, patient's motivation and compliance plays important role in treatment outcome.

The one-visit apexification has several advantages over conventional type. Once the initial infection has resolved, an osteoinductive material can be placed at the root apex to achieve an apical plug. Thereafter, obturation and permanent restoration may be placed. The tooth would subsequently be reviewed periodically for healing. Patient's motivation and compliance plays a minimal role in treatment outcome.

Current osteoinductive materials recommended in apexification are calcium hydroxide and mineral trioxide aggregate (MTA) (4). Calcium hydroxide is manufactured in two forms, powder and paste. Calcium hydroxide powder is manufactured in pure form while paste, its non-setting form, has vehicle and additives added to increase ease of application into the canals. Calcium hydroxide powder has a pH of 12.6 while paste is slightly lower (5). The exact mechanism is still unclear. The periapical tissues in contact with calcium hydroxide, develop a superficial three-layer necrosis resulting from chemical injury due to the high pH (6). This area of necrosis causes irritation and stimulates the defense and repair mechanism leading to mineralisation. The high pH of calcium hydroxide also stimulates mineralisation through activation of tissue enzymes like alkaline phosphatase (ALP) (7, 8).

MTA is mainly composed of Portland cement, bismuth oxide and calcium sulphate dehydrate. It is known for its osteoinductive potential (9-15). MTA has an initial pH of 10.2 and sets at pH 12.9 and remains constant thereafter (16). The high pH activate ALP in the surrounding tissues as in calcium

hydroxide. However, the exact mechanism of action differs due to the composition of the materials. Ionic dissociation of MTA releases hydroxyl ions O^{2-} from tetrahedron silicate and calcium oxide. These larger hydroxyl ions may be less effective against the bacterial cytoplasmic membrane (17), hence, it has a poorer antibacterial property and lower inflammatory response to the surrounding tissues compared to calcium hydroxide.

In brief, the cellular events following an insult/ injury in hard tissue formation generally undergo the following sequence; chemotaxis, proliferation, differentiation, mineralisation of hard tissue matrix and cessation of hard tissue formation activity (18). The presence of ALP is indicative of cells in differentiation phase. ALP liberates free phosphate ions into the organic matrix. These free phosphate ions react with calcium ions in the blood stream to form calcium phosphate precipitate. Calcium phosphate precipitate is a molecular unit of hydroxylapatite (19). ALP is a hydrolytic enzyme with strong relationship to the process of mineralization and a known marker for hard tissue forming cells, osteonectin and osteopontin (20-26). The optimal pH for its activation is between 8.6 and 10.3 (27). ALP is inhibited at a pH of 11.9-12.3 (28).

Though calcium hydroxide has been traditionally used in apexification, no study has been conducted to evaluate its ALP activity. Therefore, the purpose of this study was to assess the ALP activity of MG-63 cells on currently recommended osteoinductive materials used in apexification namely calcium hydroxide powder (CH), paste (CHP) and MTA.

MATERIALS AND METHODS

Cell culture

MG-63 cell line (American Type Culture Collection; ATCC number: CRL-1427) was obtained from a bone tissue of a 14 year-old Caucasian male. These cells were grown in modified Eagle's Minimal Essential Medium (EMEM). The growth medium was modified EMEM supplemented with 10% heat-inactivated fetal bovine serum while the maintenance medium was modified EMEM supplemented with 2% heat-inactivated fetal bovine serum. The cells were grown in $75cm^3$ sterile tissue culture flasks (Nunc, Germany) and incubated in a humidified atmosphere at a temperature of 37°C in 5% CO^2.

Extract preparation

The test materials used in this study were calcium hydroxide powder Pulpdent®, calcium hydroxide paste, Pulpdent® Tempcanal and grey MTA, ProRoot™. The dental materials were mixed according to their manufacturer's instructions under sterile conditions. With CH special precautions were taken, a fresh batch was purchased and the weighed material was preheated with a Bunsen burner to ensure calcium carbonate present was converted into calcium hydroxide.

The material extract was prepared by adding 5mls of maintenance medium for every 1g of test material, forming the stock (29). The concentration of test materials used was 20mg/ml (10il). The control used in the experiment was 10il modified EMEM medium without 2% heat-inactivated fetal bovine serum. 96-well microtitre plate containing material extracts were seeded with 100il of suspended MG-63 cells at 1 x 10^6. Incubation periods were 0.25h, 0.5h, 1h, 24h, 48h and 72h. Three microtitre plates were prepared for the selected durations for each material. The experiment was repeated thrice. The microtitre plates were then placed into an incubator at 37°C in 5% CO^2. At the end of the stipulated durations, BCIP-NBT (5-Bromo-4chloro-3-indolyl phosphate, toluidine salt-Nitro blue tetrazolium chloride) Liquid Substrate System (Sigma Aldrich) was used to detect alkaline phosphatase.

30µl of labeling agent was added into wells in a dark-hood as the labeling agent was photosensitive. Each microtitre plate was wrapped in an aluminium foil and incubated at 37°C in 5% CO^2 for 45 minutes. ELISA reader (Dynatech MR5000, Guernsey Channel Island) was used to record the absorbance at 410nm. The absorbance measured correlated to the number of viable osteoblast cells present. Data obtained were recorded and compiled.

RESULTS

ALP activity of MG-63 cells has been summarized in Figure 1. The null hypothesis was that the test materials do not exhibit increased ALP activity. Overall analysis indicated significant interaction between test materials and control and that duration was a factor. Test materials were subsequently, paired and analysed for initial (0.25h, 0.5h and 1h) and delayed response (24h, 48h and 72h). Survival analysis comparing multiple samples statistics, Cox's proportional hazard regression model was used to analyse data and significance level was set at $p \leq 0.05$.

During the initial response (Figure 1), CH showed the highest ALP activity followed by CHP and MTA. CH and CHP had similar downward trend while MTA showed a spike in ALP activity at 30 minutes. Only interaction between CH and MTA was significant ($p=0.0047$).

During the delayed response (Figure 1), CHP exhibited a sudden increased activity between 24 and 48h. CH showed a steep drop from 24 to 48h but ALP activity increased thereafter while MTA showed a drop in ALP activity after 48h. The interaction between CH and CHP was significant ($p=0.0510$)

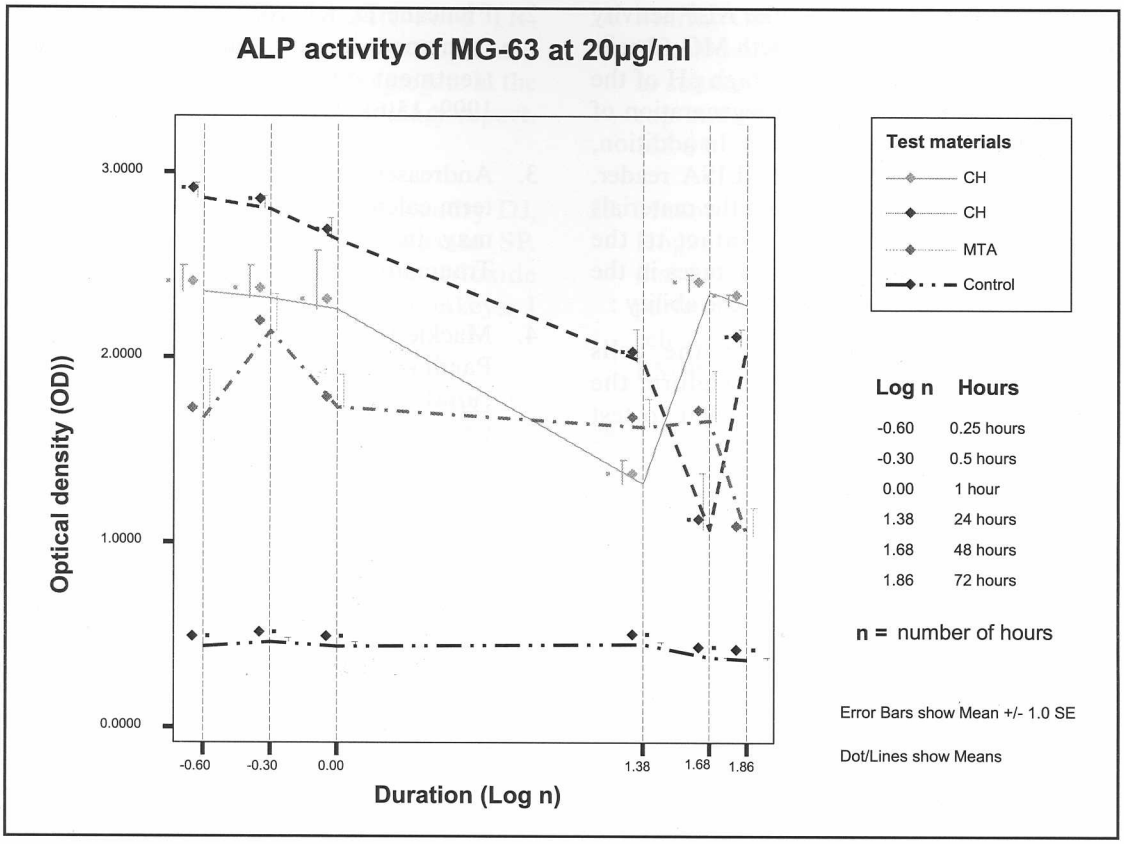

Figure 1. ALP activity of MG-63 of three materials and control.

and duration was a factor (p=0.0180). However, with CHP and MTA, the interaction between the materials was not significant but duration was a factor (p=0.0110). The interaction between CH and MTA was not significant.

DISCUSSION

Todar (30) stated that 10% FBS contained growth factors that stimulate cell proliferation when added into the flask containing cells. These cells would proliferate exponentially for three or four days until some stimulatory components of the serum was exhausted or depleted. The cells then slip into Go phase (resting) even though the nutrients are still present in the medium.

In this study, maintenance medium was used. Therefore cells entered Go phase and stayed dormant for several days. On induction, the cells in Go phase entered active proliferation and subsequently, cellular differentiation (31). It was noted that after 15 minutes, the number of viable cells was high and ALP activity remained high during the first hour. This may be indicative of cells being in differentiation phase (20). Therefore, the reaction observed in Figure 1 was most likely induced by the materials. Hence, all three materials have shown some osteoinductive potentials. This was in agreement with previous cellular and animal studies (12, 13, 32-42).

During the first hour (initial response phase), ALP activity of CH and CHP were similar. This may be due to their similar material composition. Both CH and CHP had aqueous vehicles and their biological action was determined by the rate of ionic dissociation of Ca^{2+} and OH^- ions (43). CH was mixed in sterile distilled water while CHP, a propriety product, contained methylcellulose and other additives. The vehicle used likely altered the behaviour of CHP. Also *in vitro*, ionic diffusion was also affected by the buffer substances in the culture medium (44). Both these factors contributed to CHP's lower pH (45) thus its reduced effectiveness when compared to CH.

During the delayed response phase (24-72 hours), there existed constraint of nutrients, accumulation of inhibitory metabolites and biological space. Due to the limited resources available the cells might have slipped into cell death phase (46). By 48 hours, the local environment might not have been conducive for continued cell growth. This was shown by the downward trend by the control cells in Figure 1. Interestingly, between 24-72 hours, cell differentiation had begun to accelerate for CHP and CH. CHP showed higher ALP activity compared to CH and MTA. Further studies should be carried out to investigate this phenomenon.

This present study investigated the ALP activity when material was in direct contact with MG-63 cells as per clinical application. The initial high pH of the materials may damage the cells and regeneration of cells through induction may be limited. In addition, the material may interfere with the ELISA reader. Therefore, a valid means of separating the materials from the cells while maintaining contact to the materials would have been ideal. Advantages in the ability to remove the materials include the ability :

- to replace fresh medium for the cells (continuous culture) and therefore the increase the duration of assessment to test materials beyond 48 hours.
- to practise recommendations by Denizot *et al* (47). Thus enabling a more accurate quantitation by ELISA reader without the interference of the material.

Only one *in vitro* study conducted with MG-63 cells found no significant increase in ALP activity when MTA was introduced (36). This was not in agreement with present study. However, another researcher (48) found that MTA induced ALP activity in gingival and PDL fibroblasts harvested from patients during removal of the third molar.

CONCLUSION

This study concluded that calcium hydroxide powder, paste and MTA induced ALP activity in MG-63 cells. Clinically, calcium hydroxide induces healing, unfortunately, the duration for formation of the calcific barrier is however prolonged (2, 4, 49) with a possibility of increased risk in tooth fracture with long term usage (3, 50). Clinical application of MTA in non-vital immature teeth allows the affected tooth to be restored endodontically almost immediately after achieving an apical plug (setting time approximately four hours). Beyond the apical plug, osteoinductive potential of MTA promotes undisturbed formation of the calcific bridge (35, 51).

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