Incorporation of anti-inflammatory agent into calcium hydroxide pulp capping material: An *in vitro* study of physical and mechanical properties

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The aims of this study were to investigate the release of fluocinolone acetonide from an experimental pulp capping material containing fluocinolone acetonide (PCFA) and compare some physical and mechanical properties with Dycal[®]. The PCFA is a hard-setting calcium hydroxide cement composed of 50 mmol/L fluocinolone acetonide. Conditioned media from the setting material was collected for determination of fluocinolone acetonide release by high performance liquid chromatography and pH measurement by pH meter. The setting time, compressive strength, disintegration, and acid soluble arsenic content were measured according to ISO 3107:2004. Dycal[®] was used as control. Fluocinolone acetonide could release at a range of suitable concentrations from PCFA. The pH, setting time, and acid soluble arsenic content of PCFA were significantly higher than those of Dycal[®]. The compressive strength and disintegration of PCFA were comparable to control. PCFA may be considered as an alternative in pulp capping of inflamed dental pulp tissue.

Keywords: Calcium hydroxide, Dycal, Anti-inflammatory agent, Fluocinolone acetonide, Pulp capping

INTRODUCTION

Direct pulp capping or partial pulpotomy is a method of treatment in which the exposed dental pulp is covered with a material protecting the pulp from additional injury and permitting healing and repair. Materials used in vital pulp treatment must be placed in contact with the pulp tissue^{1,2}, so it should be nontoxic and biocompatible with the tissue. The pulp capping materials should also provide good sealing ability^{3,4}. The major problems with vital pulp therapy are the unpredictable severity of the inflammatory condition of the underlying tissue and variable long-term clinical success^{5,6}. Chronic inflammation in dental pulp tissue may lead ultimately to degenerative pulp response or pulp necrosis. To prevent these complications, the anti-inflammatory agents have been used for treatment of deep carious lesion or pulpal exposure in permanent teeth with a mature apex⁷⁻¹⁰. Anti-inflammatory medication mixed with a drug delivery vehicle would be an effective direct pulp-capping material for caries-exposed pulp tissue¹¹.

Anti-inflammatory agents, such as corticosteroids, has ability to inhibit inflammatory processes such as inhibition of hyperemia and edema, pain reduction, and presumably induction of pulpal healing^{7-10,12-14}. The use of topical corticosteroids in vital pulp treatment was first reported more than 50 years ago by Rapoport and Abramson⁹, with 80–93% success in pulp capping procedures. However, a commercial product (Ledermix[®]; Lederle Pharmaceuticals, Division of Cyanamid, Wolfratshausen, Germany) containing both 1% triamcinolone acetonide and 3.21% demethylchlortetracycline in a zinc oxide-eugenol base, led to unpredictable and frequently unfavorable

results^{10,15-17}. This might be because uncontrolled release of the steroids which cause unpredictable long-term success and side effects. Recently, a number of evidence has shown the benefits of steroid usage for reduction inflammation and stimulation of pulp healing^{7,8,12,18}. Thus, short-term application of a corticosteroid to inflamed dental pulp can be considered to resolve inflammation and consequently promote healing¹⁸.

Fluocinolone acetonide is a synthetic corticosteroid commonly used for topical application in the management of dermatologic disorders and oral vesiculoerosive lesions¹⁹⁻²¹. The effects of fluocinolone acetonide on skin treatment are concentration-dependent. High concentrations of fluocinolone acetonide inhibited epidermal mitotic activity, but low concentrations slightly increased this activity²². Increased proliferation of cultured human skin fibroblasts was noted over a wide range of concentrations²³. Recently, a wide range of concentration of fluocinolone acetonide (0.1–10 µmol/L) demonstrated positive effects on human dental pulp cell proliferation, and both fibronectin and type I collagen synthesis¹⁸. With the known anti-inflammatory effect, it may have some potential in stimulation of healing of dental pulp tissue before dentin bridge formation. Successful use of this agent in pulp treatment, however, requires careful control of corticosteroid release. Research interest has focused on the incorporation of fluocinolone acetonide into the pulp capping agent to promote healing of inflamed pulp tissue. The purposes of this study were to investigate the release of fluocinolone acetonide from an experimental pulp capping agent containing fluocinolone acetonide (PCFA) and compare some physical and mechanical properties between PCFA and commercial calcium hydroxide cement.

MATERIALS AND METHODS

Preparation of PCFA

The components of PCFA, a two-paste calcium hydroxide cement containing base and catalyst, are shown in Table 1. All components of the PCFA and their roles as a pulp capping material are the same as the commercial hard setting calcium hydroxide cement (Dycal[®], Dentsply Inc., Milford, DE, USA), except fluocinolone acetonide which was added into PCFA as another active component. The fluocinolone acetonide powder (Fluocinolone acetonide, FARMABIOS S.R.L., Gropello Cairoli, Italy) was dissolved in dimethyl sulfoxide solution (DMSO; CHROMASOLV[®] Plus, Sigma-Aldrich Corp., St. Louis, MO, USA) to obtain a 10, 20, 30, 40, 50, 75, 100, and 150 mmol/L concentration before incorporation into the catalyst. The PCFA was stored at 4°C before use. Equal amount of the base and catalyst were mixed and allowed to set at room temperature to form hard setting calcium hydroxide cement. Dycal[®] was used as a control (except in the *in vitro* release assay), and prepared in accordance with the manufacturer's instructions.

In vitro release of fluocinolone acetonide

The *in vitro* release of fluocinolone acetonide assay was modified from Haesslein and co-workers.²⁴⁾ The samples were mixed and immediately put into cylindrical plastic molds (5-mm-height, 8-mm-diameter). Before complete setting, 1 mL of deionized (DI) water was poured onto the surface of each specimen. All specimens were kept at 37°C. The conditioned media was collected at specified time intervals (4 h, 8 h, and 1, 2, 3, 5, 7, 14, 21, and 28 days, and monthly afterwards) and replaced with the same amount of fresh DI water. The media samples were collected until the results showed a constant rate of release. The collected supernatant was stored at 2–8°C until analysis. The amount of released fluocinolone acetonide was determined by high performance liquid chromatography (HPLC) system equipped with a UV-VIS detector (Shimadzu, Model SPD-10A, Shimadzu Corp., Kyoto, Japan). In a typical run, 50 µL of samples were analyzed on a reverse phase C18, 5 µm column (250 mm×4.6 mm) (Inertsil[™], GL Sciences Inc., Tokyo,

Japan). A water-methanol mixture (30:70 by volume) delivered at a flow rate of 0.7 mL/min was used as mobile phase. Fluocinolone acetonide was detected at a wavelength of 238 nm and an approximate retention time of 11 min. Fluocinolone acetonide standards were prepared from a stock solution of fluocinolone acetonide in DMSO (4.52 mg/mL) by dilution with DI water. The concentration of standard was proportional to the integrated area of the corresponding peak in UV absorbance. The calibrated standard curve was linear ($R^2 > 0.99$) over the recorded concentration range of fluocinolone acetonide between 1 to 100 µmol/L. Various concentrations of fluocinolone acetonide (10 to 150 mmol/L) were loaded into the cements. The amount of fluocinolone acetonide release was recorded, and a suitable concentration of fluocinolone acetonide loading was selected for further investigation. Ten experimental samples were prepared for each group. The cement without fluocinolone acetonide was used as a control. The release of fluocinolone acetonide was calculated and recorded in µmol/L.

pH measurement

The method of pH measurement was modified from Gençay and colleagues²⁵⁾. Each specimen was mixed and placed in a cylindrical stainless steel mold with 2-mm-height and 4-mm-diameter. Then, the mold and cement was pressed between two microscopic glass slides. The samples were allowed to set in room temperature. Each sample was taken out of the mold and placed into a separate vial, containing 10 mL DI water. The samples were stored at 37°C, and pH measurement was done at 1, 3, 24, 48, 72, and 168 h (7 days) after the incubation. The pH value was measured by a digital pH meter (Orion Research 420A, Orion Research Inc., Boston, MA, USA), which connected to a glass electrode (Orion Ag/AgCl 91 Series electrode, Orion Research Inc.). The pH meter was calibrated with buffer solutions (Orion buffer solution, Perfect buffer 10, Orion Research Inc. [pH<7,00; pH<11,00]) before each experiment. Ten samples were prepared for each group. The pH values were shown as mean±standard deviation compared with the control.

Table 1 Experimental pulp capping material base and catalyst composition

	Quantity (%)
Base	
1,3-Butylene glycol disalicylate	43.00
Zinc oxide	9.00
Titanium oxide-calcium phosphate	31.00
Calcium tungstate	17.00
Iron oxide pigment	0.1
Catalyst	
Calcium hydroxide	51.00
Zinc oxide	9.23
Zinc stearate	0.29
Ethyl toluene sulfonamide + Fluocinolone acetonide	39.48

Determination of setting time

According to International Standard ISO 3107:2004, the cylindrical specimens were prepared from stainless steel molds with 2-mm-height and 10-mm-diameter. The mold was placed on a flat glass plate and filled with the mixed material. The molds and tested materials were placed on a metal block in a controlled chamber (37°C and 95% relative humidity) within 60 s from started mixing. A flat-ended indenter needle (100 g) was lowered vertically onto the surface of the cement. The indentation was immediately made at 15 s intervals in different area. The needle tip was cleaned before each indentation. The setting time was recorded as the period of time from the start of mixing to the time when the needle failed to completely penetrate the whole depth of material. Ten samples were tested for each group.

Determination of compressive strength

The method used in this study was carried out according to ISO 3107:2004 standard. Specimens were prepared from stainless molds with 4-mm-height and 6-mm-diameter. The cement was put in the mold and placed on a metal plate within 1 min after mixing. Another metal plate was placed on top and both plates were squeezed together. The whole assembly was transferred to the cabinet (37°C and 95% relative humidity) within 2 min after the end of mixing. One hour after the completion of mixing, flat surfaces of the specimen were prepared at 90° to the long axis. The specimen was removed from the mold immediately and examined for voids or chipped edges. Any defective specimens were discarded. Ten specimens were used for the experiment in each group. The diameter of the cylinders was measured and recorded. The specimens were immersed in distilled water and maintained at 37°C for 24 h, then conditioned in distilled water at 23°C for 15 min prior to test. The flat ends of each specimen were placed between the platens of universal testing machine (Instron, Model 8872; Instron corp., Canton, MA, USA). The compressive load was applied along the long axis of the specimen using cross-head speed of 1.00 mm/min until fracture occurred. Then, the maximum force was recorded. The compressive strength was calculated from the relationship between surface area and load.

Determination of disintegration

The disintegration of test materials was examined according to ISO 3107:2004. Specimens were prepared from split stainless steel rings with 1.5-mm-height and 20-mm-internal diameter. The mold was placed on a thin cellulose acetate sheet backed by a flat glass plate. A stainless steel wire (0.25-mm-diameter) was inserted through the split ring at least 10 mm into the ring. The cement and Dycal® (as a control group) were mixed and filled in the ring, then, covered with another flat glass plate faced with a sheet of cellulose acetate, and firmly pressed together. After the complete set (approximately 3 min after the start of mixing), the molds and plates were placed in the cabinet maintained at 37°C and 95% relative humidity. Then, the specimens and attached

wires were carefully removed from the ring. The excess cement was removed from the edge of the specimen disc, and lightly brushed to remove any loose material from the surface. The specimens were weighed and net weight of cement (total weight minus with stainless steel wire) was recorded. Two of the sample discs were placed in a 100 mL beaker. The discs were submerged immediately by pouring 50 mL of distilled water into the beaker. The specimens were suspended by the wire without contact to each other and to the beaker. Then, the beaker was sealed with plastic wrap and aluminum foil. After immersing the discs for 24 h at 37°C, the specimens were removed from the water. Their surfaces were rinsed with 2 mL of distilled water. The surfaces were gently dried with clean absorbent paper. The specimens were then stored in a desiccator for 24 h and reweighed. The final net mass of cement was then recorded. The disintegration was calculated as percentage of the original weight. Ten samples were prepared from each group.

Determination of acid-soluble arsenic content

The method used in this study was examined according to ISO 3107:2004. The set experimental cement and Dycal® (as a control group) were pulverized and passed through a 75 µm (200 mesh) sieve. Two grams of the sieved powder was dispersed in 30 mL of water with 10 mL of 37% hydrochloric acid (Hydrochloric acid fuming 37%, Merck KGaA, Darmstadt, Germany). The mixture was maintained at 37°C for 1 h, and filtered. The total arsenic content of the sample was determined with the atomic absorption microscopy (AAnalyst 800, PerkinElmer Inc., Shelton, CT, USA). Ten samples were prepared from each group.

Statistical analysis

All data were analyzed by using a statistical analysis program (SPSS version 17.0, SPSS Inc., Chicago, IL, USA). The significant difference between material properties was determined by Student's *t*-test ($p < 0.05$). The data of setting time was discrete data. Therefore, it was shown as median and analyzed by Mann-Whitney *U* test ($p < 0.05$).

RESULTS

In vitro release of fluocinolone acetonide

The amount of released fluocinolone acetonide was determined by HPLC and UV detection directly from the release media. The HPLC method allowed for the detection of fluocinolone acetonide at a retention time 11.0 min (Fig. 1a). The amounts of released fluocinolone acetonide from various concentrations of the experimental materials were presented in Fig. 1b. Cements with 50 mmol/L fluocinolone acetonide loading could release fluocinolone acetonide at a concentration of 50 µmol/L from 50-mm²-surface area which is equal to 1 µmol/L/mm². Therefore, 50 mmol/L of fluocinolone acetonide loading was selected for further investigation.

The cumulative release of fluocinolone acetonide from the PCFA was investigated in the *in vitro* release

Fig. 1 Release of fluocinolone acetonide from experimental cement. (a) Chromatogram obtained from HPLC and peak of fluocinolone acetonide (arrow); (b) Selection of 50 mmol/L fluocinolone acetonide loading from various doses of experimental pulp capping materials; (c) Cumulative release of fluocinolone acetonide; and (d) Fluocinolone acetonide concentration released over each different periods of time. Data are represented as means±standard deviation.

study over 6 months. The result was shown in Fig. 1c as the mean±SD of the released fluocinolone acetonide. Fluocinolone acetonide was dramatically released over the first 72 h (Fig. 1d). This early rapid release also affected the cumulative release of fluocinolone acetonide, especially in the first 5 days as shown in Fig. 1c. After 1 month, fluocinolone acetonide was released from the PCFA at an almost constant rate of 0.029–0.021 μmol/L/day.

pH measurement

The pH of the experimental material and Dycal® became increased from neutral after 1 h. The pH of PCFA was significantly higher than that of Dycal® at every time interval ($p<0.05$). The pH of Dycal® and PCFA ranged between 9.80 to 10.86 and 10.57 to 11.72, respectively. The pH of both materials remained stable after 72 h as shown in Fig. 2.

Determination of setting time, compressive strength, disintegration, and amount of acid-soluble arsenic content

The physical and mechanical properties of the experimental material were examined and the results shown in Fig. 3. The setting time of Dycal® and PCFA ranged from 60 to 90 s and 115 to 165 s, respectively. The median setting times of Dycal® and PCFA respectively

Fig. 2 pH values of PCFA and Dycal®. Data are represented as means±standard deviation at each time interval. *Data show statistically significant differences between materials tested in corresponding method.

were 75 and 150 s (Fig. 3a). The compressive strength of Dycal® and PCFA were 17.09±2.91 and 15.11±1.25 MPa, respectively (Fig. 3b). The disintegration of Dycal® and PCFA were 3.13±0.43% and 3.15±0.22% respectively (Fig. 3c). The compressive strength and disintegration of Dycal® and PCFA were not significantly different

Fig. 3 Physical and mechanical properties of experimental cement (PCFA) and control (Dycal®): (a) Setting time; (b) Compressive strength; (c) Disintegration; and (d) Acid-soluble arsenic content. Data of setting time is represented as median. The remaining data are represented as means±standard deviation. *Data show statistically significant differences between materials tested in corresponding method.

($p < 0.05$). The acid-soluble arsenic content of Dycal® was less than 0.05 mg/kg, whereas of PCFA was 0.21 ± 0.02 mg/kg (Fig. 3d). The setting time and acid soluble arsenic content of PCFA were significantly higher than those of Dycal® ($p < 0.05$).

DISCUSSION

The use of anti-inflammatory agents for vital pulp therapy began in the 1960s. However, there have been questions about the safety of these medicaments. Fluocinolone acetonide has been demonstrated to be successful and safe to use in treatment of oral vesiculoerosive lesions^{19,21}. A limit number of evidence reported the positive proliferative effect of low range of concentrations of fluocinolone acetonide^{22,23}, but the inhibitory effect for cell growth is one of the well-known adverse effects of topical glucocorticoids^{26,27}. Thus, application of fluocinolone acetonide in vital pulp therapy was discouraged, and it should be used with cautions²⁷.

Our recent study showed the appropriate concentrations of fluocinolone acetonide in promotion of early healing response of pulp cells were $0.1\text{--}10\text{ }\mu\text{mol/L}$ ¹⁸. Fluocinolone acetonide, therefore, was incorporated into the calcium hydroxide hard setting cement at the selected concentration (50 mmol/L) to release the suitable

concentration. The chromatogram of HPLC showed several peaks of components of the PCFA, but most of them were too small. We could confirm the availability and stability of the released fluocinolone acetonide by comparison with the peak of the standard agent at the same retention time. The *in vitro* release study demonstrated fluocinolone acetonide was able to leach out from the material over a period of 6 months. The release of fluocinolone acetonide concentration, ranging from 0.1 to $1\text{ }\mu\text{mol/L}$, was found within the first 5 days. This period corresponds to the early phase of wound healing, the inflammatory and proliferative phases which start immediately and a few days after the treatment, respectively²⁸⁻³⁰. Since 0.1 to $10\text{ }\mu\text{mol/L}$ fluocinolone acetonide has stimulatory effect on cell proliferation and extracellular matrix formation¹⁸, the initial burst release of fluocinolone acetonide should be beneficial to healing. However, the activity of released fluocinolone acetonide is still not known and has to be confirmed by further *in vitro* and *in vivo* studies. Cell culture system can be used to study the effect of materials, with and without the release of fluocinolone acetonide, on cell proliferation, RNA and protein synthesis, or other cellular activities. The animal study must be done to confirm the healing of dental pulp tissue after capping with the PCFA or control. After 1 month,

fluocinolone acetonide released from the PCFA at approximately 0.029–0.021 $\mu\text{mol/L/day}$. A minimal amount of fluocinolone acetonide released from the material is preferred over the long-term period to prevent both systemic effects of the steroid such as hyperglycemia, glaucoma, adrenal insufficiency, and local effects such as delayed wound healing and risk of microbial proliferation^{26,27,31,32}.

Calcium hydroxide products have been accepted as a standard material for conservative treatment of dental pulp exposures due to their therapeutic and biological potential, their property of stimulating formation of sclerotic and reparative dentin, as well as protecting the pulp against thermal stimuli¹. The success obtained with calcium hydroxide as a pulp capping agent is related to its high alkalinity and it is suggested the rise in pH was the most important factor conducive to pulp healing^{33–37}. The conditioned media obtained from Dycal[®] had pH values of 9.80–10.86 over the 168-h (7 days) duration of this assay. This finding was in agreement with previous reports^{25,38–43}. The conditioned media obtained from PCFA also maintained high alkaline levels, with pH values of 10.57–11.72, which were significantly higher than those of Dycal[®] during the 168-h (7 days) duration. This difference may be due to the different amount of hydroxyl ions released from each material. The higher pH value may have some advantages in neutralization of the acid at the site of inflammation, antibacterial effect, and stimulation of the repair and dentin bridge formation in the exposed pulp^{25,36,42,44}.

The ISO standard for a pulp capping material has not been developed. Therefore, ISO 3107:2004 was selected as a guideline for evaluation of the material properties. The setting time of the PCFA (150 s) was longer than that of Dycal[®] (75 s). Earlier studies reported a longer setting time of Dycal[®] (145 s) which may be due to the different methods used among the studies^{39,45}. The setting time of the hard-setting calcium hydroxide is also affected by the room temperature. Therefore, the longer setting time of PCFA will be adequate for using at higher temperature condition. The compressive strength of PCFA (15.11 ± 1.25 MPa) was comparable to Dycal[®] (17.09 ± 2.91 MPa). Previous studies reported the compressive strength of Dycal[®] in a range of 14.5 to 36 MPa^{43,46,47}. However, the compressive strength of both cements was much higher than the minimal strength required by the ISO 3107:2004. They are strong enough to resist the average stress of 10.5 N/mm², which is applied through an amalgam condensation cycle⁴⁸. Disintegration of the PCFA (3.16%) was equal to that of Dycal[®] (3.13%) and was comparable to the previous reports of Dycal[®] (2.7 to 6.76%)^{39,49}. The solubility of the cement may be beneficial for the release of hydroxyl ions and fluocinolone acetonide, but it may cause the dissolution of the material and leave space under a restoration⁴⁹. Due to the low compressive strength, disintegration and lack of adhesion to dentin, the cement should be used as a thin layer and covered by other base or liner material which provides a good seal and prevent the direct contact with acid etching procedure⁵⁰. The

arsenic contamination in experiment cement (PCFA) was determined by the standard method. The test material was dispersed in acid until the contaminated arsenic was dissolved into the acid solution. The acid-soluble arsenic content of PCFA (0.21 mg/kg) was higher than that of Dycal[®] (0.05 mg/kg). This arsenic content in PCFA was approximately ten-fold below the maximal dose according to the ISO 3107:2004 standard and far below the lethal dose (1.5 to 500 mg/kg of body weight) administered orally in human^{51,52}.

Mineral trioxide aggregate (MTA) has been proven to be successful in vital pulp therapy. It can stimulate thick dentinal bridge and appears to have particular promise as a pulp capping material^{53–55}, even in cases of irreversible pulpitis^{56–58}. However, MTA has slow setting time and high cost. Some authors have reported the similar result between MTA and calcium hydroxide when used in human teeth^{33,59,60}. Although MTA is popular as a pulp capping material at present, it has different compositions and properties to the calcium hydroxide cements. Dycal[®] was chosen as the control in this study because of the similarity of type, composition, and usage. After the incorporation of fluocinolone acetonide into the experimental pulp capping material, fluocinolone acetonide could release and some physical and mechanical properties were not different from Dycal[®]. Therefore, PCFA may be considered as an alternative material in treatment of vital pulp or vital pulp therapy, instead of conventional calcium hydroxide cements. However, effect of fluocinolone acetonide or PCFA to dental pulp tissue is still not known. Further *in vitro* and *in vivo* experiments are required to confirm the biologic effect of this agent or material. Pulp tissue healing and quality of dentin bridge should be evaluated by comparison with MTA in an *in vivo* study before this material is introduced for clinical use.

CONCLUSIONS

The results from this study have shown that fluocinolone acetonide could release at suitable concentrations from the experimental anti-inflammatory calcium hydroxide cement. The pH, setting time, and acid-soluble arsenic content were higher than Dycal[®], but the compressive strength and disintegration were not different from Dycal[®].

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