行政院國家科學委員會專題研究計畫 成果報告

探討根管充填劑對人類骨細胞的作用機轉(第 2 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 97-2314-B-040-022-MY2
執 行 期 間 ：98 年 08 月 01 日至 99 年 07 月 31 日
執 行 單 位 ：中山醫學大學牙醫學系（所）

計 畫 主 持 人：黃富美
共 同 主 持 人：張育超

報 告 附 件 ：出席國際會議研究心得報告及發表論文

處 理 方 式 ：本計畫可公開查詢

中 華 民 國 99 年 11 月 11 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

（計畫名稱）

探討根管充填劑對人類骨細胞的作用機轉

計畫類別：■ 個別型計畫　□ 整合型計畫

計畫編號：NSC －96－ 2314－B－022－MY2

執行期間： 97 年 8 月 1 日至 99 年 7 月 31 日

計畫主持人：黃富美

共同主持人：張育超

成果報告類型(依經費核定清單規定繳交)：□精簡報告 ■完整報告

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

■出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢

□涉及專利或其他智慧財產權，□一年■二年後可公開查詢

執行單位：

中 華 民 國 99 年 10 月 31 日

ABSTRACT

Histologic investigations have demonstrated that root canal sealers can induce mild to severe bone resorption. Alkaline phosphatase (ALP) is a membrane-bound glycoprotein, which is one of the osteogenic differentiation markers considered to indicate the formation of new bone. The aim of this study was to investigate the effects of an epoxy resin-based sealer AH26, a zinc oxide-eugenol-based sealer Canals, and a paste sealer N2 on the expression of ALP in human osteoblastic cell line U2OS cells. **Methods**: Freshly mixed materials were filled in glass rings and eluted in 10 ml of culture medium for 1 day. Subsequently, various dilutions (final dilution: 1/2, 1/4, and 1/8) of these extraction media were prepared for this study. Cytotoxicity was measured by the almar blue dye assay. Gene expression of ALP was examined by using reverse transcription-polymerase chain reaction. ALP activity was further evaluated by using substrate assay. **Results**: The results showed that AH26, Canals, and N2 were cytotoxic to U2OS cells in a concentration-dependent manner ($p<0.05$). The exposure of U2OS cells to AH26 and N2 resulted in the down-regulation of ALP mRNA gene expression ($p<0.05$). ALP activity was significantly suppressed by three root canal sealers ($p<0.05$). **Conclusions**: The inhibition of ALP expression may play an important role in the pathogenesis of root canal sealers-induced periapical bone destruction.

INTRODUCTION

Root canal sealers may have a significant influence on the apex because their potentially toxic ingredients can initiate irritation of the periapical tissues and may interfere with the healing process. The materials used for endodontic procedures must be chosen with care so that potential periapical reactions can be avoided or reduced. Ideally, root canal sealers should be biocompatible and have satisfactory physico-chemical properties.

Currently, root canal sealers are available based on various formulae such as epoxy resin, calcium hydroxide, and zinc oxide-eugenol. The irritative effects of root canal sealers have been evaluated by histopathologic examinations of the tissue response. Unfortunately, several studies have shown that most commercially available root canal sealers can induce mild to severe inflammatory alteration within apical tissues leading to bone destruction. Recently, our studies have shown the activation of inflammatory mediator cycloxygenase-2, inflammatory cytokines interleukin-6 and interleukin-8, antioxidant enzyme heme oxygenase-1, proteolytic enzymes gelatinases and tissue type plasminogen activators, and osteolytic marker receptor activator of NF-κB ligand may play an important role in root canal sealers-induced periapical inflammation. Despite the above evidences, the pathogenesis of root canal sealers-induced periapical bone destruction still remains to be elucidated.

Alkaline phosphatase (ALP) is one of the most frequently used biochemical markers of osteoblast activity. ALP is present on the plasma membrane fragments of the osteoblasts. Osteoblasts are considered the cells primarily concerned with providing physical barriers and structural components in the periapical tissues. The purpose of this study was to determine an epoxy resin-based sealer AH26, a zinc oxide-eugenol-based sealer Canals, and a paste sealer, such as N2 could influence the functions of osteoblasts. The effects of root canal sealers on human osteoblastic cell line U2OS cells were determined through measuring cell viability, ALP mRNA expression, and ALP activity.

MATERIALS AND METHODS

Sample preparation

The test materials were shown in table AH26 (De Trey Dentsply, Switerland), Canals (Showa Pharmaceutical Co., Tokyo, Japan), and N2 (Indrag-Agsa, Losone, Switzerland). Under aseptic conditions the root canal sealers were mixed according to the manufacturer's instructions. Samples were prepared as follows: freshly mixed materials were filled in glass rings (4 mm height and 10 mm in diameter) and set for 24 h at 37°C in a humidified chamber. Each test specimen was eluted in 10 ml of culture medium at 37°C for 1 day in a 5 % CO_2 air atmosphere. The extraction media were then collected into sterile syringes at the end of this period and passed through a 0.22 μm filter. Subsequently, various dilutions (final dilution: 1/2, 1/4, and 1/8) of these extraction media were prepared to be used in this study.

Cell culture

U2OS cells (American Tissue Type Collection HTB 96) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10 % fetal calf serum (FCS) (Sigma Chemical Co., St. Louis, MO), 100 μg/ml of streptomycin, 100 mg/ml of penicillin at 37°C in humidified incubator under ambient pressure air atmosphere containing 5 % CO_2. Confluent cells were detached with 0.25 % trypsin and 0.05 % EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every 3rd day.

Cytotoxicity Assay

Almar blue is an oxidation-reduction indicator for eukaryotic cells. It uses as an indicator to assess cellular metabolic activity. It is a developed extension of the cytotoxicity assay based on the reduction of tetrazolium salts by the mitochondrial cytochromoxidase system. Cells were seeded 5×10^4 cells per well into 24-well culture plates and incubated for 24 h. The culture medium was replaced with fresh DMEM and various elutes of root canal sealers. After trypsinization, 25 μl of alamar blue dye (Alamar Biosciences Inc., Sacramento, CA, USA) were added to each well for 2 h at 37°C. The colorimetric determination was done at 570 nm and 600 nm on a plate reader (CytoFluor 4500, Millipore, Bedford, MA, USA). The inhibition of

mitochondria activity of the experimental groups were divided by the control and expressed as a percentage of control.

Reverse-transcriptase polymerase chain reaction

Total RNA was prepared by using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) and following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco Laboratories, Grand Island, NY, USA). The reaction mixture was diluted with 20 μl of water, and 3 μl of the diluted reaction mixture was used for the polymerase chain reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Taq DNA polymerase. Amplification was performed at 25 cycles for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 30 cycles for ALP in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C. The sequences of primers used were as follows:

A) GAPDH Forward: 5'-TCCTCTGACTTCAACAGCGACACC-3'

Reverse: 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3'

B) ALP Forward: 5-TGGAGCTTCAGAAGCTCAACACCA-3

Reverse: 5-ATCTCGTTGTCTGAGTACCAGTCC-3

When the samples were probed for ALP mRNA production by RT-PCR, a 454 bp band for ALP was noted. These bands were consistent with the size as designed by primers. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Measurement of ALP activity

The cell lyates were sonicated on ice bath, centrifuged at 1500 g for 5 min, and measured the ALP activity in supernatant by ALP assay mixtures containing 0.1 M 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, and 8 mM *p*-nitrophenyl phosphate disodium. After

incubation at 37°C for 30 min, the reaction was stopped with 0.1 N NaOH and the absorbance was read at 405 nm. The OD of control cells was considered to be 100 %. The relative ALP activity of U2OS cells was calculated by the formula: (OD of experimental sample/OD of control cells) x 100 %. Each value was expressed as the mean ± SD.

Statistical analysis

Triplicate experiments were performed throughout this study. All assays were repeated three times to ensure reproducibility. The significance of the results obtained from control and treated groups were statistically analyzed by paired Student's *t*-test.

Conclusion

This is the first attempt to evaluate the role of ALP mRNA expression and ALP activity in human osteoblastic cells. Data from our in vitro experiments showed that root canal sealers were capable of suppressing ALP activity in human osteoblastic cells. This suggests that one of the pathogenic mechanisms of apical bone destruction in vivo may be the inhibition of ALP by resident cells in response to root canal sealers challenge. However, care should be taken to reduce the possibility of periapical irritations from inadvertent extrusion of the root canal sealers in clinical treatment until their side effects are more thoroughly studied.

Table I. Composition of test materials according to manufacturers' descriptions

Material	Components	Ingredients
AH26	Powder	Bismuth (III) oxide Hexamethylene tetramine Ag TiO_2
	Liquid	Bisphenol-A-diglycidylether
Canals	Powder	ZnO Rosin Barium sulfate Bismuth subcarbonate
	Liquid	Clove oil Peanut oil
N2	Powder	ZnO Bismutnitrate Bismutcarbonate Paraformaldehyde TiO
	Liquid	Eugenol Peanut oil Rose oil

RESULTS

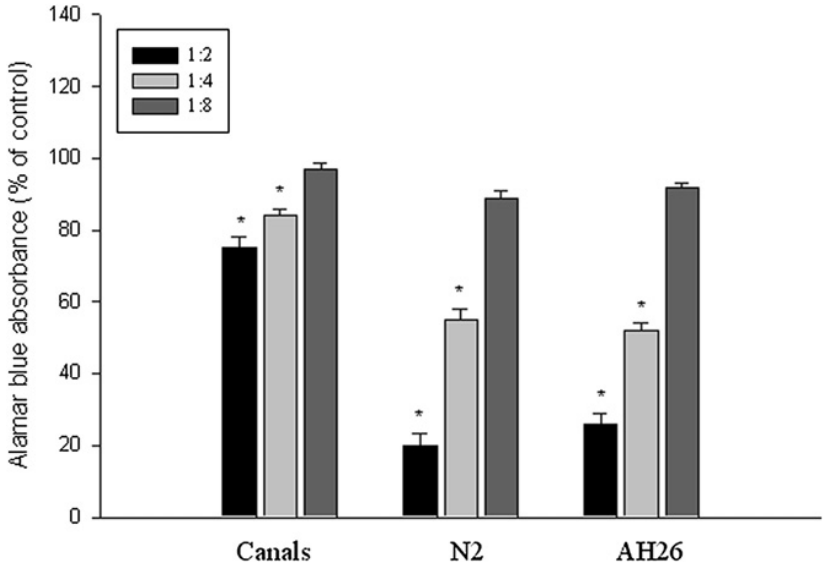

Fig. 1 Cellular toxicity measured by using alamar blue dye on U2OS cells after exposure to various elutes of root canal sealers for 24 h. Results are expressed as percentage of absorbance relative to the untreated control. Data are shown as mean ± SD. * denotes significant differences from control values with $p<0.05$.

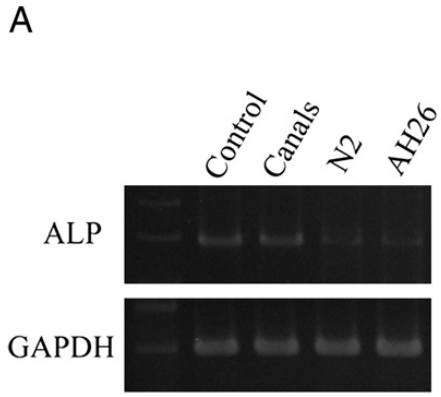

Fig. 2 (a) Expression of ALP mRNA gene in various root canal sealers-treated U2OS cells by RT-PCR assays. GAPDH gene was performed in order to monitor equal RNA loading.

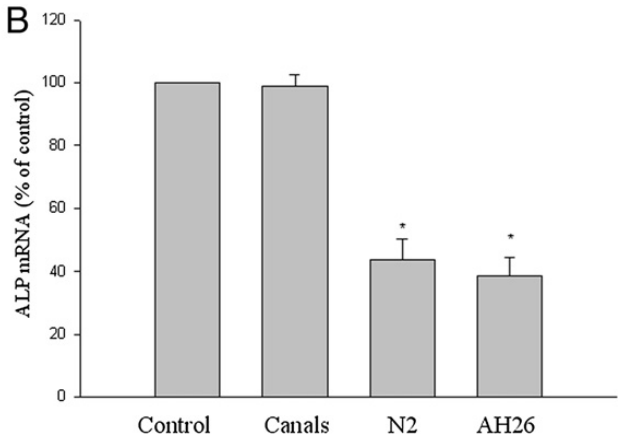

Fig. 2 (b) Levels of ALP mRNA treated with the AH26, Canals, and N2 were calculated, as measured by AlphaImager 2000. The results were averaged from three independent experiments. * denotes significant differences from control values with $p<0.05$.

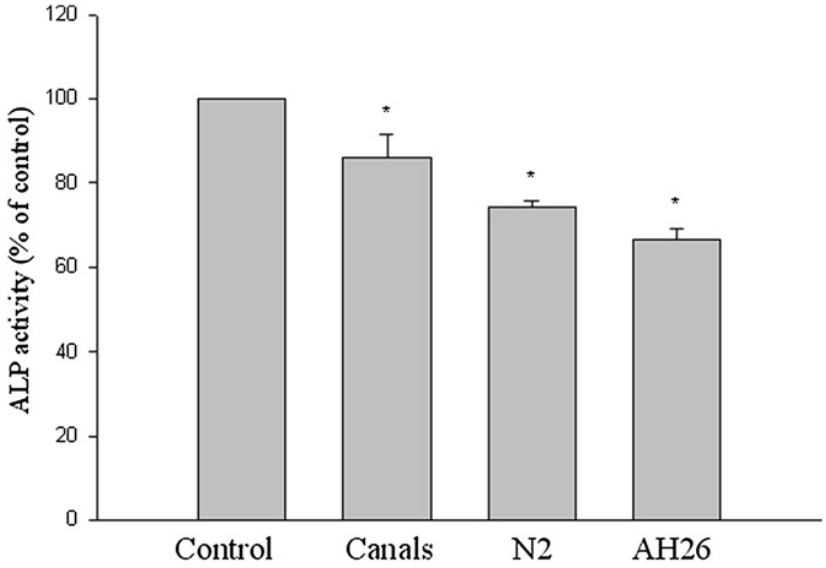

Fig. 3 Effects of AH26, Canals, and N2 on the ALP activity in U2OS cells. The percentages of ALP activity in the presence of root canal sealers relative to that in the control are shown. The results were averaged from three independent experiments. * denotes significant differences from control values with $p<0.05$.

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

99 年 10 月 31 日

報告人姓名	黃富美	服務機構及職稱	中山醫學大學牙醫學系 教授
時間 會議 地點	99 年 7 月 14 日至 7 月 17 日，西班牙（Spain）巴塞隆納(Barcelona)	本會核定補助文號	NSC 97-2314-B-040-022-MY2
會議名稱	（中文）第 88 屆國際牙醫研究學會年會 （英文）88th General Session & Exhibition of the international Association for Dental Research		
發表論文題目	（中文）根管充填劑會抑制人類骨細胞中鹼性磷酸酶的表現 （英文）Inhibition of ALP by root canal sealers in human osteoblasts.		

報告內容應包括下列各項：

一、參加會議經過

今年的國際牙醫研究學會年會於西班牙巴塞隆納舉行，本屆年會會期正值巴塞隆納觀光旺季，雖然天氣十分炎熱但是觀光人潮不斷湧進，治安十分不良，很多參加牙醫研究學會會員相機皮包被偷，所幸我們組團前往，大多團體行動在會期結束後一切平安。

論文發表發表形式分為 oral presentation、poster presentation、poster discussion 三種。筆者今年報告的論文題目為 “Inhibition of ALP by root canal sealers in human osteoblasts” 以 poster presentation 方式發表。ALP(鹼性磷酸酶）在成骨細胞（製造骨頭的細胞）中含量豐富，成人 ALP（鹼性磷酸酶）的參考值為 96~284 U/L。兒童期因骨骼正在成長，成骨細胞中 ALP 很活躍，血清中 ALP 之濃度可高至成人之兩~三倍，因此可檢測鹼性磷酸酶在骨細胞之變化表現，以了解各種類根管充填劑對造骨細胞之影響。所有組織學的研究，都顯示各種類型的根管充填劑都會對根尖周圍組織造成不同程度的發炎反應或引起輕微到嚴重骨吸收。 鹼性磷酸酶是造骨細胞膜上醣蛋白，這些細胞被破壞或增殖時，鹼性

磷酸酶濃度就會增加，因此鹼性磷酸酶的活性常被當作骨形成與骨吸收的標記。本研究擬偵測環氧樹脂類根管充填劑 (AH26)和鋅氧化物-丁香油酚基的(Canals 和 N2) 根管充填劑對鹼性磷酸酶在人類骨細胞 U2OS 細胞中的表達。 方法：依廠商說明指示均勻調拌根管充填劑，取一定量放入圓形玻璃管，馬上浸泡在 10cc 培養液中浸泡 24 小時，收集浸泡釋出液（最後稀釋： 1/2、 1/4、 1/8）做為實驗用。Almar 藍色是細胞生存能力研究的一種新穎的染料。本研究利用 Almar 藍色染料法測量根管充填劑對細胞毒性作用。 再使用逆轉錄-聚合酶鏈反應 RT-PCR 偵測鹼性磷酸酶基因的表達，以評估檢測鹼性磷酸酶的活性。 結果：顯示 AH26、Canals 和 N2 對 U2OS 細胞毒性，與根管充填劑濃度成正相關（$p < 0.05$）。 AH26 和 N2 對 U2OS 細胞抑制鹼性磷酸酶的基因表達下降 ($p < 0.05$)。 AH26、Canals 和 N2 根管充填劑顯示抑制鹼性磷酸酶活性 ($p < 0.05$)。結論：鹼性磷酸酶表達的抑制作用可能在根管充填劑誘導根尖周圍骨細胞發炎破壞中扮演著重要的角色。

二、與會心得

國際牙醫研究學會已受到政治的介入，臺灣無法自己成立單獨的 division，目前歸在 South-East Asian Division，而中共自前年加入即自成 China Division，政府宜儘速因應。本次我們組團，團員包括台大、中國、中山、高雄等四家牙醫學系研究成員參加，可算是最愉快一次經驗，平常大家都忙，利用這次機會除了作國際間交流，也交換國內學術心得；本次盛會收穫良多，吸取了許多寶貴的經驗及目前研究的新方向，對於往後的研究裨益良多，再此亦非常感激國科會予以經費補助參與此次國際牙醫研究學會年會。

國科會補助計畫衍生研發成果推廣資料表

日期:2010/11/03

國科會補助計畫	計畫名稱: 探討根管充填劑對人類骨細胞的作用機轉
	計畫主持人: 黃富美
	計畫編號: 97-2314-B-040-022-MY2　　學門領域: 牙醫學

無研發成果推廣資料

97 年度專題研究計畫研究成果彙整表

計畫主持人：黃富美	計畫編號：97-2314-B-040-022-MY2
計畫名稱：探討根管充填劑對人類骨細胞的作用機轉	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	無
		研究報告/技術報告	0	0	100%		無
		研討會論文	0	0	100%		無
		專書	0	0	100%		無
	專利	申請中件數	0	0	100%	件	無
		已獲得件數	0	0	100%		無
	技術移轉	件數	0	0	100%	件	無
		權利金	0	0	100%	千元	無
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	無
		博士生	0	0	100%		無
		博士後研究員	0	0	100%		無
		專任助理	0	0	100%		無
國外	論文著作	期刊論文	1	0	100%	篇	研究計畫成果已發表在 SCI 期刊(J Endod 2010；36:1230－1233)
		研究報告/技術報告	0	0	100%		無
		研討會論文	0	0	100%		無
		專書	0	0	100%	章/本	無
	專利	申請中件數	0	0	100%	件	無
		已獲得件數	0	0	100%		無
	技術移轉	件數	0	0	100%	件	無
		權利金	0	0	100%	千元	無
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	無
		博士生	0	0	100%		無
		博士後研究員	0	0	100%		無
		專任助理	0	0	100%		無

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估 ■達成目標 □未達成目標（請說明，以 100 字為限） □實驗失敗 □因故實驗中斷 □其他原因 說明：
2. 研究成果在學術期刊發表或申請專利等情形： 論文：■已發表 □未發表之文稿 □撰寫中 □無 專利：□已獲得 □申請中 ■無 技轉：□已技轉 □洽談中 ■無 其他：(以 100 字為限)
3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)