行政院國家科學委員會專題研究計畫 成果報告

自行改良配方之三氧礦化物(mineral trioxide aggregate)其物理化學性質與生物學效應研究
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（計畫名稱）

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中文摘要

臨床上，牙齒覆髓劑、斷髓劑或根尖充填劑乃是將生物相容或誘導之材料放置於暴露之組織，許多藥物材料都曾被應用於牙齒覆髓或是斷髓，如formocresol (FC), gluteraldehyde, ferric sulfate (FS), zinc oxide eugenol, polycarboxylate cement, calcium hydroxide.，近年mineral trioxide aggregate (MTA) 或是bioactive glass (BAG)也常被應用於斷髓之治療。其中MTA 材料於根管治療領域中已廣泛的被應用多年，然而其特性上仍有缺點，如硬化時間過長、不易塑型以及成本費用過高等問題至今仍無解決。延續前次之研究計畫對於傳統斷髓藥物或材料作用於牙髓細胞後之生物效應結果，我們認為可於覆髓劑、斷髓劑或根尖充填劑材料之改良上作一改質。因此，本研究計畫目的將首先先自行合成所要之MTA 配方，並對其物性上，如硬化時間、PH 值變化等，與商品化之MTA作一系列之比較評估。將本實驗依成分不同分為四組，氧化鈣大約佔了65%，氧化矽大約佔了25%，氧化鋁大約佔了5%，剩下的5%則是由其他多種的氧化物所組成，將原料略加研磨混合，再以可程式控制之高溫爐煆燒至1400℃，持溫兩個小時後爐冷。將所得的產物先以研缽磨細，再經過球磨機六個小時的研磨，最後由mesh 的篩網過篩，得到粉末粒徑大小皆小於22 μm。研究結果如下：本研究室已可自行合成MTA材料；XRD 晶相分析經過資料庫比對後，自行製備材料部分，最強的繞射峰2θ=27.4°以及2θ=33.1°搭配其晶相為Bi2O3 (No.41-1449)；位於2θ=29.6°, 32.1°-32.72°, 34.3°, 41.2°, 47.5°的繞射峰為C3S (No. 17-0445)有關，而C2S (No. 31-0297)產物則是出現在2θ=20.5°, 21.8°, 23.2°, 32.1°~32.7°。自製的四組材料中的硬化時間，大約可以縮短至11 到13 分鐘之間。市售MTA 與四組材料在水合反應中，大約經過水合反應二十分鐘後便趨近平緩，六個小時後的pH 值也是大約在附近，與市售MTA 的差異不大。四組材料在經過水合反應一天後的拉伸強度比較。HMA 組(0.89±0.10 MPa)的拉伸強度皆小於其他三組(2.74±0.15、2.91±0.08、3.10±0.08 MPa)。結語；本次研究之結果基本物理性質與化學性質與商品化之性質相似。日後可繼續進行材料之生物相容性探討。

關鍵詞 牙齒覆髓，三氧礦化物，硬度，拉伸強度，酸鹼值

英文摘要

The procedure of the pulp capping, pulpotomy or pulpectomy is to place biocompatible material inside the canal or on exposure tissue. There are many biocompatible materials used in endodontic treatment. Such as formocresol (FC), gluteraldehyde, ferric sulfate (FS), zinc oxide eugenol, polycarboxylate cement, calcium hydroxide, these materials are commonly be used. Recently, mineral trioxide aggregate (MTA) or bioactive glass (BAG) are selected to apply in pulpotomy therapy. MTA has been used in endodontic treatment for several years, although it is a good material in endodontic treatment, but there still has some defects in its characters, such as prolong setting time, difficult build up and high cost etc. These problems still not been resolved yet. From the previous results of last project from NSC, which topic as traditional pulpotomy materials effects on primary culture pulp cell. It is supposed that there still have some rooms for these materials to be improved. Thus, the purpose of this study is to made up the different formulas of MTA by ourself, then comparing it with the commercial MTA. It is to evaluate the differences within physical and chemical properties. Material and method: The CaO、SiO_2、Al_2O、Fe_2O_3 and other designed materials will be added to mixing, burning out and grind to the desired particles. According to International organization for standardization for dental root canal sealing material (ISO 6876:2001), the material will mix and compare it with commercial MTA in the following properties：setting time, PH change, strength test, electron microscope observation, solubility test , dimension stability and X ray radiopaque test. The results showed the MTA powder can be made by our laboratory, XRD analysis showed high peak of the angle is $2\theta=27.4°$and $2\theta=33.1°$. The setting time of our MTA can reduce to 11 to 13 minutes. The pH value after material hydration is similar with the commercial MTA. The tensil strength can up to 3.10±0.08 MPa. Conclusion: The preset study showed our MTA fabrication is success at this moment. Further tests should focus on the biological effects of this MTA material.

keywords: MTA, physical and chemical property, setting time, tensil strength, pH value.

前言

當牙齒齲齒嚴重傷害至牙本質時，牙齒之牙髓可能會外露，臨床上之處置乃依診斷之輕重結果來處置，通常輕者需做牙齒覆髓(pulp capping)，或是斷髓(Pulpotomy)，嚴重時需做拔髓術(Pulpectomy)。牙齒覆髓或是斷髓乃是將生物相容或誘導之材料放置於暴露之組織，因此牙齒覆髓時會將刺激牙本質形成之物質如氫氧化鈣類材料放置於暴露處；牙齒斷髓時則會將牙冠處之牙髓去除而保有剩餘牙髓之活性，其方法不外使用藥物處理或是雷射處理。

由過去資料可發現，許多藥物材料都曾被應用於牙齒覆髓或是斷髓，這些材料包括ivory, quill, gold beater skin, oiled skin, paper, plaster or Paris, Canada Balsam, asbestos, gutta-percha, lactophosphate of lime, oxychloride, oxyphosphate, and oxyxulphate of zinc cement等。[1] 針對斷髓之藥物材料常用則有formocresol (FC), gluteraldehyde, ferric sulfate (FS), zinc oxide eugenol, polycarboxylate cement, calcium hydroxide. [2] 其中Formocresol為乳牙斷髓時常用之藥物。近年來mineral trioxide aggregate (MTA) 或是bioactive glass (BAG)也常被應用於斷髓之治療。[3 三氧礦聚合物(簡稱MTA, Mineral Trioxide Aggregate)其主要成分為tricalcium silicate, tricalcium oxide 和silicate oxide. 近年來，常被用於作根尖充填之材料。第一代之成分顏色呈灰色，第二代改良為白色(White mineral trioxide aggregate)。MTA其特性如下：具有良好之封閉效果；MTA混合之initial pH值為10.2，三小時後變為12.5，它具有好的compressive strength。[4] 根據材料廠商之敘述，MT 具有下列之適應症： (1) 可用於作Apexification ； (2) 作為根管不小心穿通(perforation)之管壁修復材料；(3)髓腔底部穿通之修復；(4)修復牙根吸收現象；(5)作為根尖逆充填(root end filling)；(6) 牙齒覆髓(pulp capping)。過去研究， 發現MTA對骨細胞之具生物相容性，細胞DNA fragmentation 則未發現；對於 細胞受刺激後之胞內訊號傳遞機發現Erk kinase 有明顯之表現。[5] MTA使用根尖手術後之根尖充填結果，許多報告顯示有良好之反應。但是MTA目前於商品上之價格偏高，且硬化時間(long setting time)上較久，不易塑形為其主要缺點，另外它容易因環境之改變而影響其物性之表現。Lee等研究發現於PH5環境下，MTA之物性結構與水合反應(hydration)會受到影響。[6] 顯示MTA材料仍有其改良之空間。

過去傳統上治療牙齒斷髓術時會採用 Formocresol (FC) 或是paramonochlorophenol (PMC)，此技術應用於牙齒斷髓治療已經有一段時間，其理由為這些材料於根管治療時具有 antibacterial activities.。[7,8] FC 是否具有細毒性，於過去許多學者研究報告中仍然是有存疑的。[9-11] Formocresol 當中之一種成分 formaldehyde 則被認為是具有致癌。[12-15] 近年研究發現，利用彗星分 析(comet assay) FC 之基因毒性，以 mosue lymphoma cell line 為目標結果發現 FC 並 不會造成 DNA 之斷裂。[16]於 Sprague Dawley rats 之大白齒研究則發現 FC 的組織 學效應出現萎縮(atrophy)、發炎(Inflammation)和纖維(fibrosis)

現象。[17] 然而於　臨床上之治療，使用 FC 處理牙髓時，它仍被認為可以得到高的成功率。[18,19] 近　年有研究發現以 ferric sulphate 取代 FC 來治療斷髓，研究結果指出 ferric sulphate　毒性較 FC 低，長追蹤之臨床成功率與 FC 相同。[20] Hydroxyapatitie 為一種含有　無基質最多之成分，它亦常被用作為骨頭移植之材料。[21,22] Dental Plaster　當未與水混合之前是 calcium sulphate hemihydrate，與水混合之後則轉換成 dihydrate form。而 Plaster of Paris 則可能是第一種可吸收之骨頭移植物被用於作全身性之研究材料。[23] 當與 hydroxyapatite 混合之後可作為自體移植(allograft)之材料。[24] Dycal 因其具有 calcium disalicylate，臨床上常用於作 liningmaterial，當材料開始硬化時，其 pH 值為 10.9，並且持續釋放 hydroxyl ion。[25,26]研究指出它對牙髓組織具有很好的相容性反應。[27]

本研究目的將首先先自行合成所要之 MTA 配方，並對其物性上，如硬化時間、PH 值變化等，與商品化之 MTA 作一系列之比較評估。

材料與方法

一、粉末製備過程

1. 將CaO、SiO_2、Al_2O_3 以及Fe_2O_3 等依照適當比例混和均勻。
2. 放入高溫爐中，設定不同溫度與加熱製程條件，如：從室溫加熱至1400 度，再持續加熱兩個小時，再降至室溫。
3. 將燒結後的產物取出，研磨成粉末。
4. 將研磨好的粉末，用孔徑大小為20μm的篩網過篩。
5. 將過篩後的粉末與石膏依照4:1 的比例，並加入所欲加入之配方材料如生長素或生長因子類（對細胞生長有助益之材料）或膠原蛋白（colleganse）與骨膠（gelatine），均勻混和。

二、基本物化理性質測試，參考International organization for standardization for dental root canal sealing material (ISO 6876:2001)之規範測試

本實驗測試步驟包括本研究室自行合成之MTA 與商品化之白色MTA。

（1） 硬化時間測試

1. 將先前準備好的自行合成之MTA 粉末，與去離子水依照3:1 的比例均勻混和。
2. 白色MTA 依照廠商建議之方法調拌混合。
3. 製作成一定型平面。
4. 每五分鐘於硬度測驗機(Gillmore needle)下測試一次，紀錄觀察是否硬化。

（2） pH 值測定

1. 將先前準備好的粉末，與去離子水依照3:1 的比例均勻混和。

2. 白色MTA 依照廠商建議之方法調拌混合。
3. 將混和好的成品，放在pH meter 上。前兩個小時，每五分鐘做一次記錄，之後每半個小時作一次記錄。

（3）**強度測試**

1. 將先前準備好的粉末，與去離子水依照3:1 的比例均勻混和。
2. 白色MTA 依照廠商建議之方法調拌混合。
3. 將混合好的成品放入模具中，硬化後取出。將硬化的柱狀物以universal
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test machine 做強度測試。

（4）**電子顯微鏡下觀察**

1. 將先前準備好的粉末，與去離子水依照3:1 的比例均勻混和。
2. 白色MTA 依照廠商建議之方法調拌混合。
3. 將混合好的成品放入模具中，硬化後取出。
4. 將上述物質於電子顯微鏡下觀察其表面變化。

（5）**energy dispersive X-ray analysis (EDXA)與XRD(X ray diffraction) 表面結晶結構成分觀察**

1. 將先前準備好的粉末，與去離子水依照3:1 的比例均勻混和。
2. 白色MTA 依照廠商建議之方法調拌混合。
3. 將混合好的成品放入模具中，硬化後取出。
4. 將上述物質於、EDXA、XRD 觀察其表面之成分比例。

（6）**溶解度測試、體積穩定度測試與X 光通透性測試**

1. 將先前準備好的粉末，與去離子水依照3:1 的比例均勻混和。
2. 白色MTA 依照廠商建議之方法調拌混合。
3. 將混合好的成品放入模具中，硬化後取出。
4. 將上述物質依照ISO 6876:20015 之規範加以測試。

叁、統計方法

所有之試驗均執行三重複試驗，將所得之數據收集後，以ANOVA 統計比較其差異，當$p<0.05$ 時訂為有統計學上差異。

結果與討論

新型根管充填材料開發

鈣矽生醫陶瓷材料粉末性質分析

由Table. 1 可以將本研究所製備出的材料粉末分為四類，主要都是以氧化鈣及二氧化矽為主體的材料，另外再加入了不同的金屬氧化物以改變其物理或是化學性質。

Table 1. 各組別成分

	A	B	C	D
CaO	▲	▲	▲	▲
SiO2	▲	▲	▲	▲
Al2O3	▲	▲	▲	▲
ZnO		▲		
MgO			▲	
Fe2O3				▲

圖 1 為本研究製備之不同成分以及市售MTA 粉末外觀圖。

從材料外觀顏色來看， HMA、HMB 及HMC 皆是帶有氧化鉍的淡黃色，而HMD則因為生料中含有氧化鐵，所以是偏黑灰色，而市售MTA 為帶有淡乳黃色的粉末。從掃瞄式電子顯微鏡的觀察中，自製粉末的顆粒大小分佈約從6μm 到10μm不等，而市售MTA 的粉末顆粒分佈則是約在3μm 到20μm 之間。XRD 晶相分析經過資料庫比對後，自行製備材料部分，最強的繞射峰2θ=27.4°以及2θ=33.1°搭配其晶相為Bi2O3 (No.41-1449)；位於2θ=29.6°, 32.1°-32.72°, 34.3°, 41.2°, 47.5°的繞射峰為C3S (No. 17-0445)有關，而C2S (No. 31-0297)產物則是出現在2θ=20.5°, 21.8°, 23.2°, 32.1°~32.7°。在自製的材料與商品化MTA 最大的差異是在2θ=12°時，自製材料有一很強的繞射峰，這是商品化MTA 所沒有的，因此推測此繞射峰或許與自製材料可大幅縮短硬化時間有關。

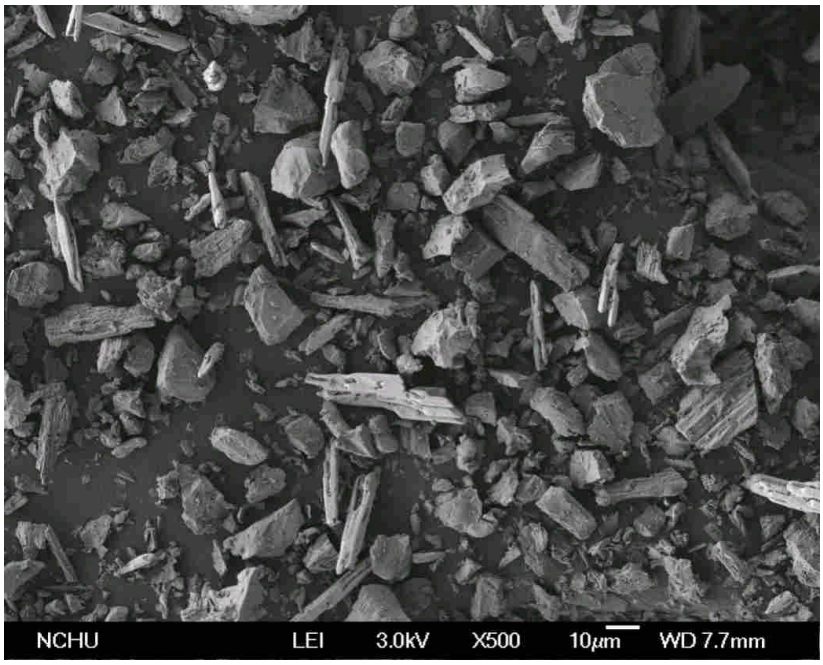


Fig. 1 (A) HMA 粉末 SEM 圖

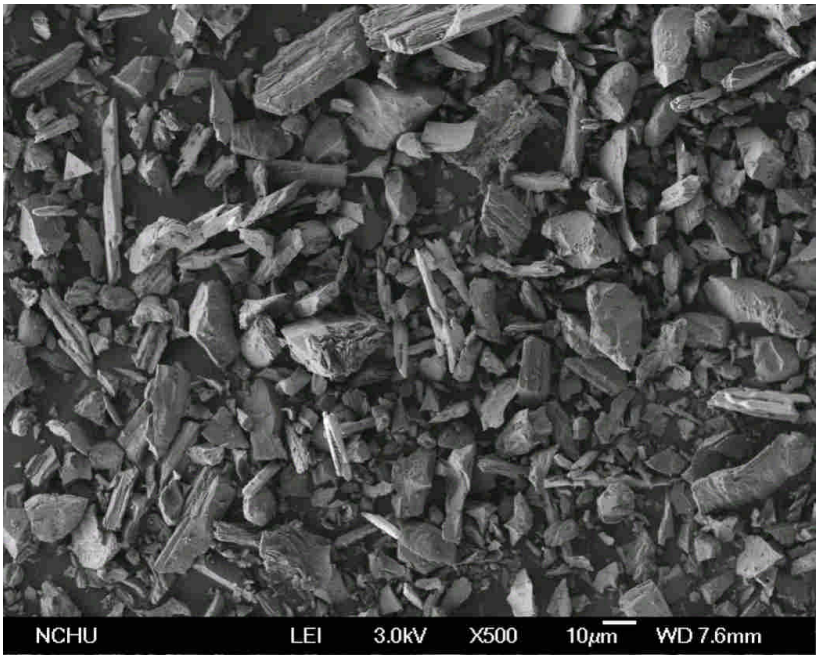


Fig. 1 (B) HMB 粉末 SEM 圖

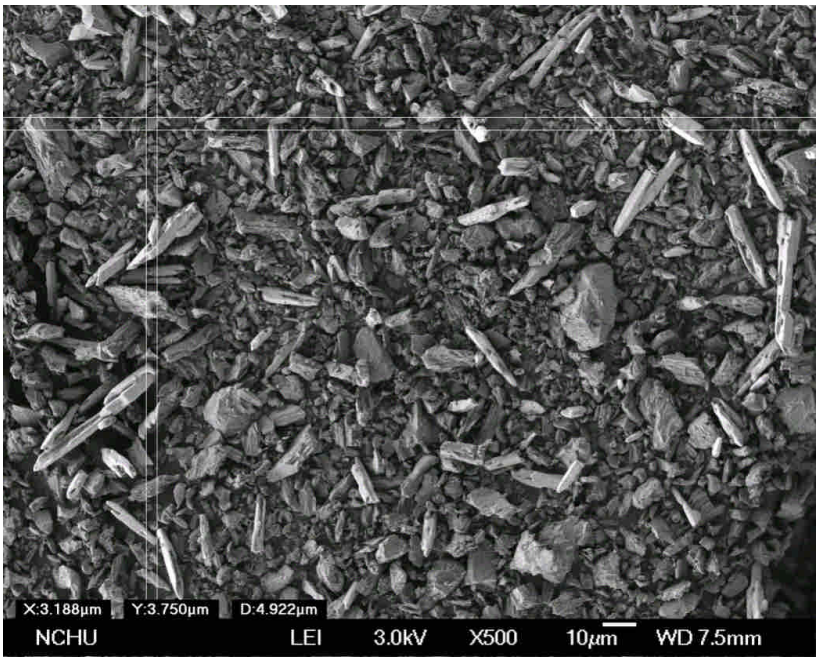


Fig. 1 (C) HMC 粉末SEM 圖

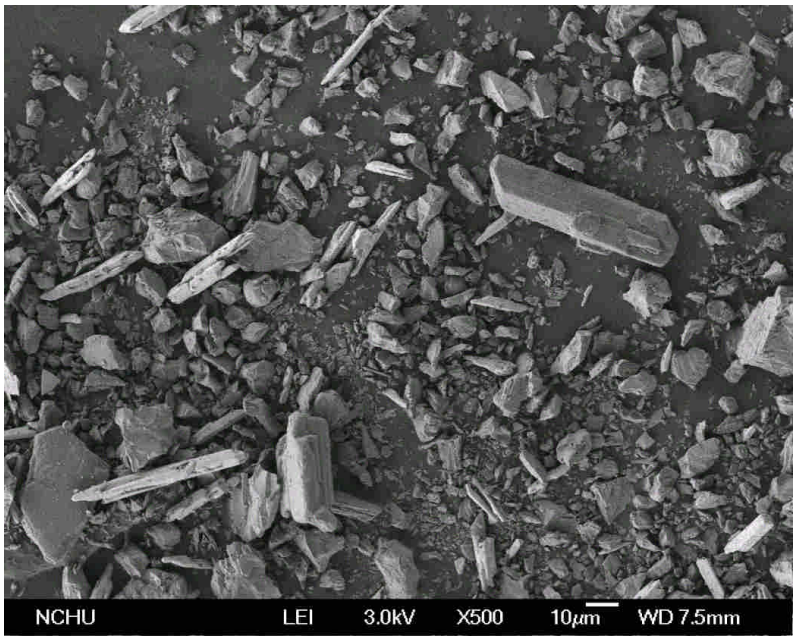


Fig. 1 (D) HMD 粉末SEM 圖

硬化時間測試

根據市售MTA 商品內的說明，其硬化時間約是165 分鐘，根據ISO 9917 的規範測試的硬化時間則約為150 分鐘左右，差異並不大。而在自製的四組材料中的硬化時間，大約可以縮短至11 到13 分鐘之間。

Fig. 2 (A) MTA 與自行製備材料粉末在XRD 的比較

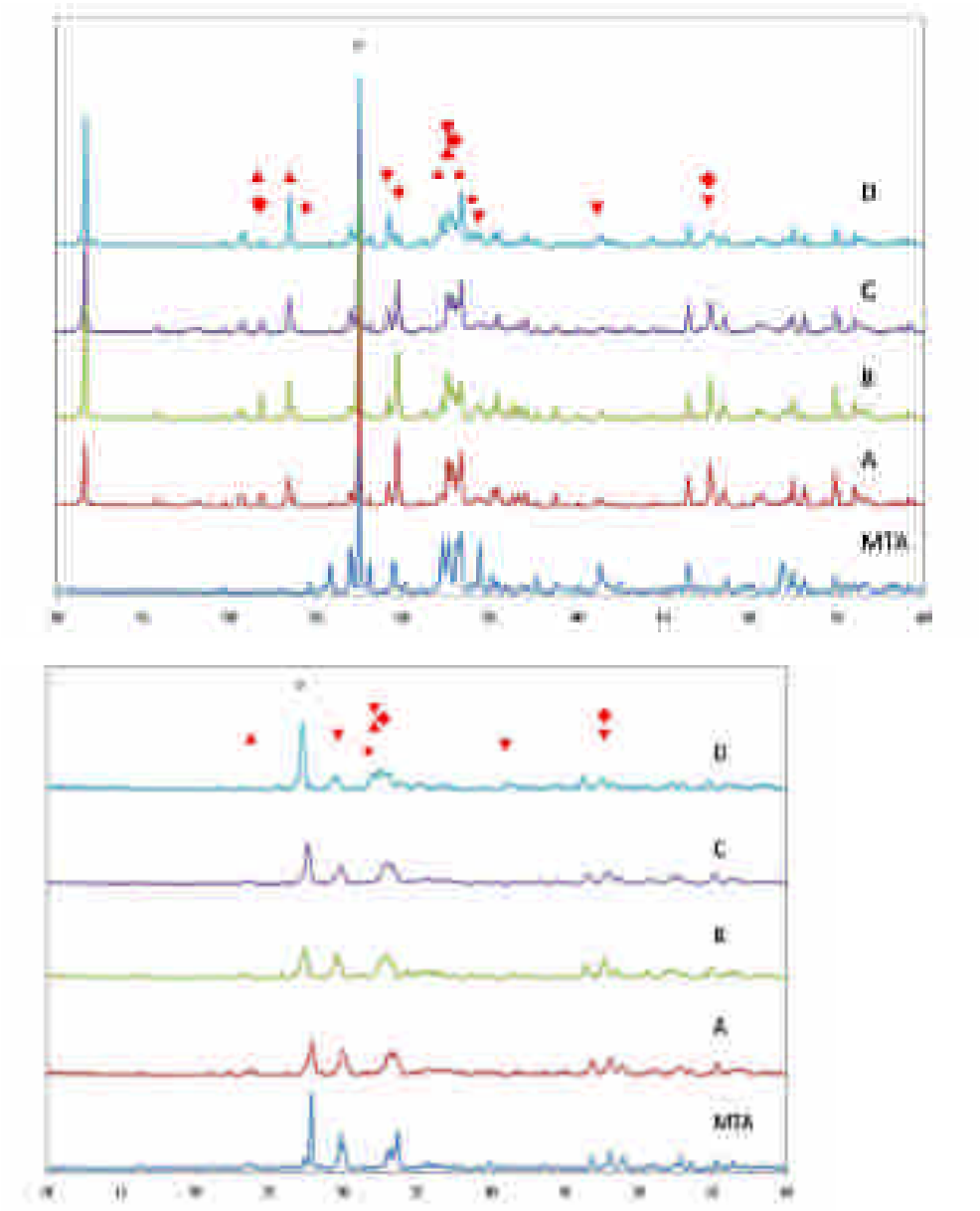


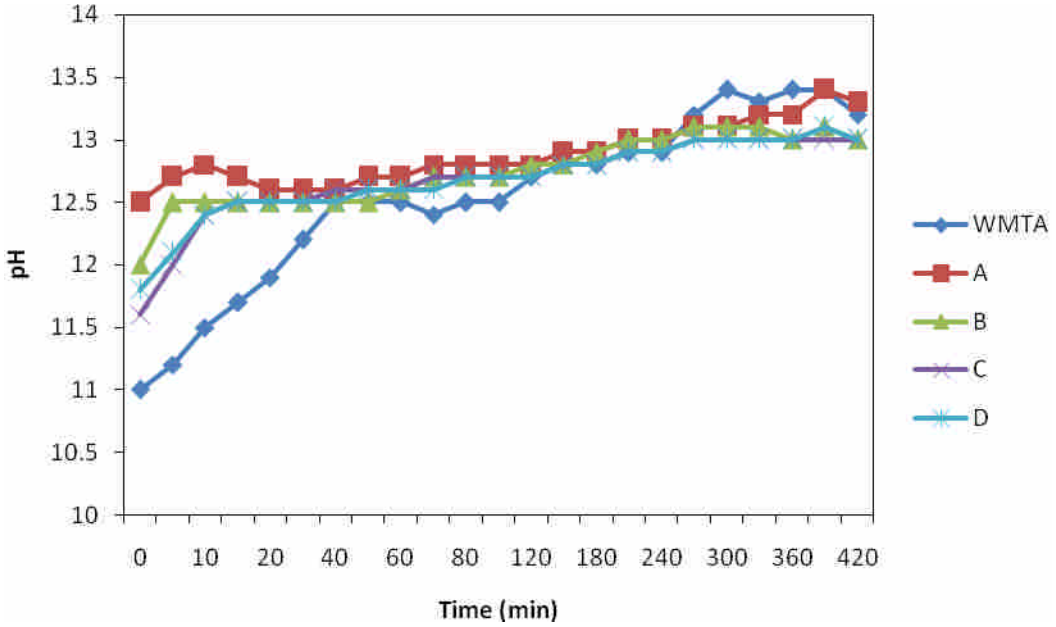
Fig. 2 (B) MTA 與自行製備材料經水合作用在XRD 的比較。

水合過程pH 值變化

Fig. 3 為市售MTA 與四組材料在水合反應中，隨著反應時間之pH 的變化曲線。在市售MTA 的部分，水合反應的前一個小時pH 值有急速上昇的趨勢，這是因為經過反應後MTA 會有氫氧化鈣的產生，而提高其pH 值，之後便開始趨

近緩和，六個小時後pH 值約在13 左右；在自製材料部分，初始的pH 值由11.至12.5 不等，但是在大約經過水合反應二十分鐘後便趨近平緩，六個小時後的pH 值也是大約在附近，與市售MTA 的差異不大。

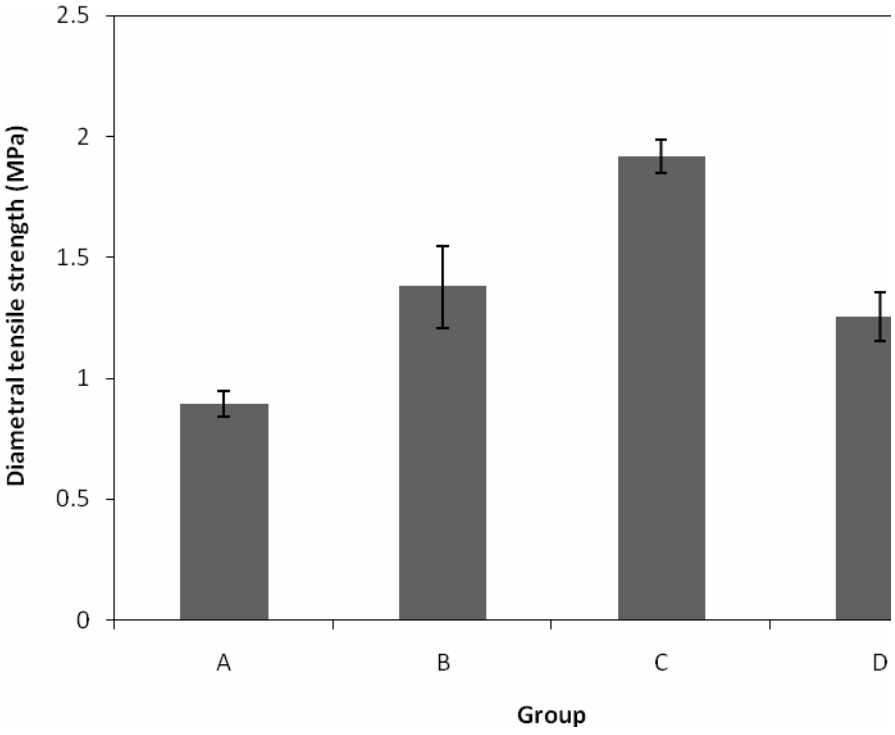
Fig. 3 MTA 與自行製備材料經由水合作用後pH 值的變化



材料拉伸強度比較

Fig. 4 為四組材料在經過水合反應一天後的拉伸強度比較。HMA 組(0.89±0.10 MPa)的拉伸強度皆小於其他三組(2.74±0.15、2.91±0.08、3.10±0.08 MPa)，而它們之間的差異便是HMA 中較其他組別少添加了5%的金屬氧化物。雖然沒有直接證據可以證明金屬氧化物的添加可使得材料瑕燒過程中增進本身的物理質，但是可以在這裡看出添加了不同的金屬氧化物，可以造成材料強度上明顯的差異。

Fig. 4 自行製備材料拉伸強度比較



浸泡測試

Fig. 5 為四組材料浸泡於SBF 中不同時間點的重量損失。
在第一天時並沒有太大的重量改變，而到了第三天各組重量約損失4-7%，接下來到一個星期便持續平緩，之後開始下降。因此推測材料在浸泡的過程中應該有其他的物質生成。
Fig. 6 為試片浸泡於SBF 環境中不同時間點的表面產物型態。在第一天中，可以發現四組皆有球狀結晶的生成，這些結晶與材料本身表面的針狀及片狀結晶構造是不相同的。並且隨著浸泡時間的增加，其表面的球狀結晶產物也有隨之增加的現象，尺寸大約都在1μm 左右。並且在HMC 組中，浸泡一天後所生成的球狀結晶，已經將試片的表面幾乎蓋滿，也可以證明鋅的介入可以促進磷灰石的沈積；而在HMB 當中，由於這一組的材料含有MgO，根據文獻指出，Mg+2 為最有效抑制氫氧基磷灰石的合成，並且會促進轉變成
β-tricalciuphosphate(β-TCP)[27-30]。

Fig. 7 為試片浸泡於SBF 環境中不同時間點的XRD 分析結果。將浸泡前與浸泡後的材料做一比較，可以發現在2θ=27.4°以及2θ=33.1°的Bi2O3 (No. 41-1449)有明顯的減少，但是在2θ=29.6°以及2θ=48.2°的繞射峰強度有顯著的增強，也代表著材料水合作用在經過浸泡後更加完全。而與HAP 相關的繞射峰2θ=25.9°，也在浸泡一天後出現，而其他與HAP 相關的繞射峰2θ=32.4°~32.8°則與C3S 及C2S

繞射峰有重疊，因此也無法判斷HAP 是否有生成。

Fig. 8 為試片浸泡於SBF 環境中不同時間點的強度比較。由圖中可以發現，浸泡一天後的強度皆有明顯的增加，與先前XRD的結果可以得知，浸泡後材料的水合作用較為完全而導致強度上升，一個禮拜後的強度則趨近平緩。從本研究中的SEM 觀察表面的結晶反應有類似HAP 的顯微球狀結構，XRD 的分析也發現低結晶性的HAP 繞射峰增加，44因此可以推論由本實驗室自製出來的四種材料，浸泡於模擬體液的環境下，可在表面產生鈣磷化合物，也具備了生物活性材料的
性質。
因此若是將這些材料應用在根管治療方面，材料的表面與體液接觸後所形成的HAP 層，推測對於吸引牙本質細胞來生長貼附會有正向的幫助。

結語

本實驗室第一次合成 MTA，並測試其物理與化學性質，結果發現與現商品化之成份特性相似，此結果可讓本實驗室繼續進行系列之生物學效應評估。

Fig. 5 自行製備材料浸泡於 SBF 中的重量損失

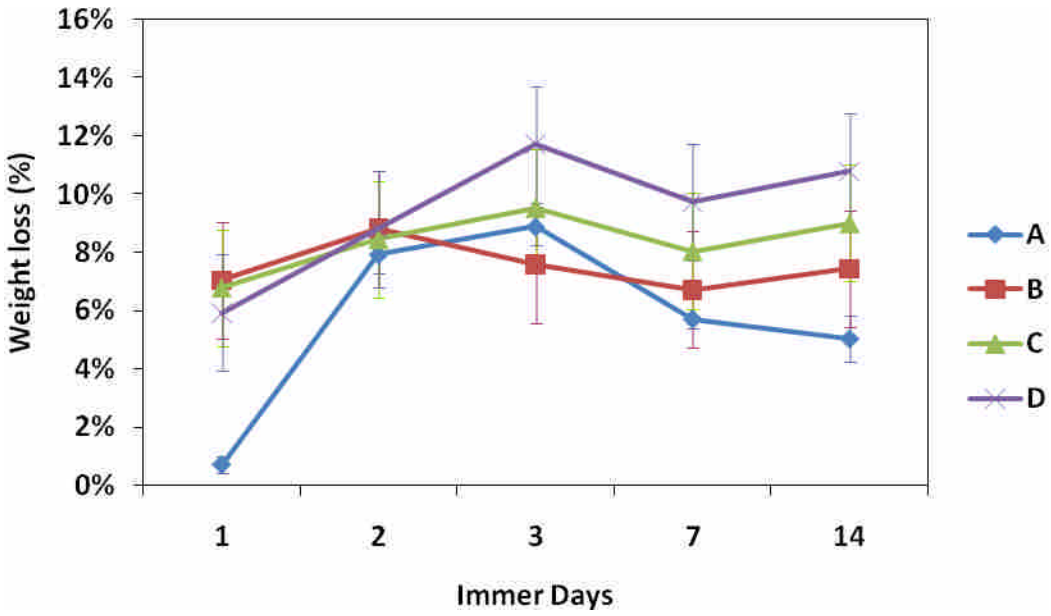


Fig. 6 (A) HMA 浸泡於 SBF 一天之 SEM 圖

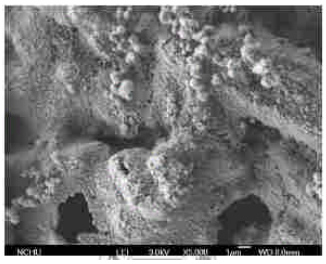


Fig. 6 (B) HMA 浸泡於 SBF 三天之 SEM 圖

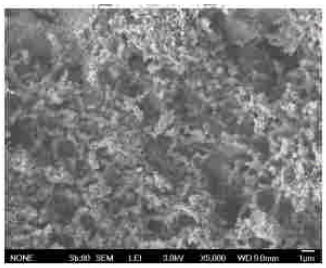


Fig. 6 (C) HMA 浸泡於 SBF 七天之 SEM 圖

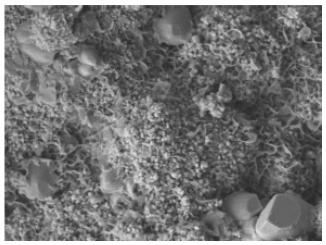


Fig. 6 (D) HMA 浸泡於 SBF 十五天之 SEM 圖

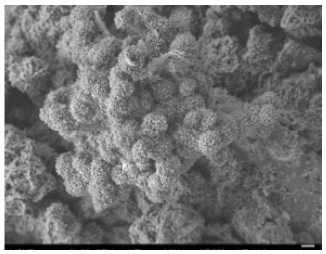


Fig. 6 (E) HMA 浸泡於 SBF 三十天之 SEM 圖

Figure 7. HMA 粉體與浸泡於 SBF 不同時間 XRD 比較

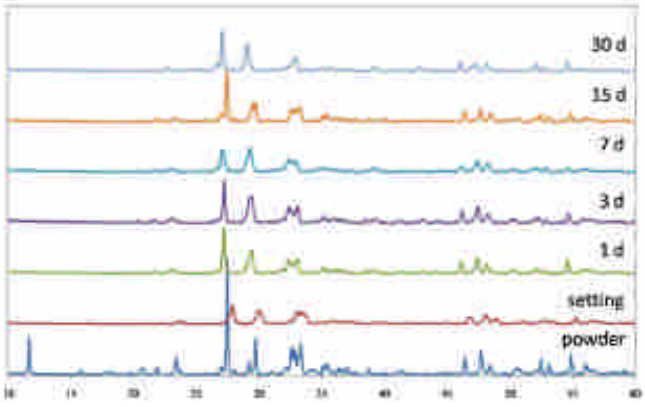
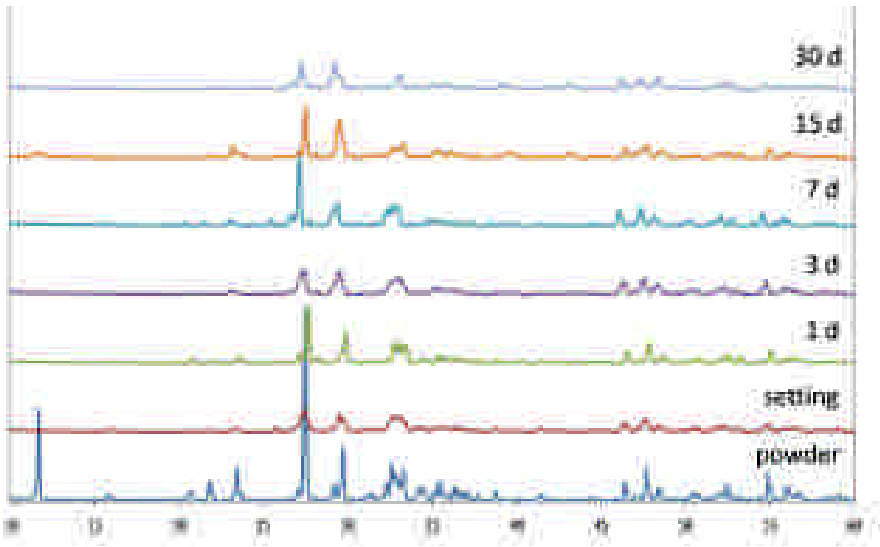


Figure 8. HMB 粉體與浸泡於 SBF 不同時間 XRD 比較



(六)計畫成果自評部份，

請就研究內容與原計畫相符程度：內容與計劃大致上相符合

達成預期目標情況：有達到本計畫的 研究目的

研究成果之學術或應用價值：已投稿至期刊現審查中。

綜合評估:

1. 由於臨床現在使用之 MTA 材質對於其硬化時間與價位均令人不滿意，因此本研究則以自行研發之方式希望能取代此，並提供臨床醫師更好且更廉價之材料。
2. 本材料之開發經ＸＲＤ測試，雖發現於 10-20 度之水合反應，材料位出現如商品化之 peak， 歸納原因，可能是由於本試驗水合後支應化放置狀態不同導致，詳細結果有待再確認。
3. 本材料之開發以解決硬化所面臨的問題，我們可以讓材料於短時間內硬化。此將可替臨床醫師解決不方便之問題。
4. 相信在接續之實驗進行，將可發現其生物相容性應是可被接受的，且對於骨組織應是有幫助的一種材料。

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出席國際學術會議心得報告

計畫編號	95-2314-B-040-022-
計畫名稱	自行改良配方之三氧礦化物(mineral trioxide aggregate)其物理化學性質與生物學效應研究 -
出國人員姓名 服務機關及職稱	黃翠賢、中山醫學大學牙醫學系教授
會議時間地點	美國 NewOrleans
會議名稱	第八十五屆國際牙醫學會學術研究大會(85th General session and Exhibition of the International Association of Dental Research)
發表論文題目	**Toxicity of pulpotomy dressing materials on L929 cells.**

一、參加會議經過

由於當地同時有美國心臟病學會大會舉辦，班機不易訂到位置，經過多次轉機終於到達此二年前才經過災患摧殘的都市，本屆國際牙醫學會學術研究大會於三月二十一日至三月二十四日於美國的紐奧爾良(NewOrleans)舉辦，此行有研究生隨者一起參加，此都市外觀感覺似乎已重建恢復，我們的到來可以刺激當地經濟，協助恢復。此屆大會參與人數人數較少，海報展是約有 3000 篇，其中不乏撤回者。參加會議中有趣參與口頭報告與專題演講的場次，聆聽人數較過去少些，挑選有興趣之主題去聆聽，並與發表者討論。晚上演講結束後與同事參加姐妹校(UAB)邀請之招待茶會。會場上有廠商之展示會，順便也一併參觀。

二、與會心得

每次參加此大會，總感到國外舉辦此大會之能力實在很足夠，反觀國內之場地與設備似乎較為缺乏，由於我國現屬於南亞洲之會員國，如有要搶辦此大會似乎機會不大，但如有機會則似乎又要擔心場地等問題。會中最讓人感覺得有趣的還是於海報論文方面展示，可以詳細的與報告者討論，分享別人的經驗。而口頭報告則因時間較短，英文能力不足者可能會吃虧，不易有太多的收穫。會場中之廠商展示，可以看到新的材料和儀器設備，可以知道當今之發展潮流。隨行中，有研究生之參與，也趁機給與教導，強調語文之重要，此大會讓後輩看到世界的研究風氣，可以刺激他們的鬥志。

國際上有許多學術大會，綜觀此會是最具學術味道，此大會是值得鼓勵有志於於研究的人員參加，除了學術上經驗之交流外，人際之交流也是參加會議之最大收穫。

Abstract

A number of pulpotomy dressing materials have been applied clinically with various rates of success. The purpose of the present study was to evaluate the toxicity of different medicaments on treated L929 cells. The puplotomy preparations were grouped as follows: 1. Zinc oxide powder, eugenol and formocresol (FC). 2. Zinc oxide powder, eugenol and glutaraldehyde(Glu). 3. Zinc oxide powder, eugenol and ferric sulfate (FeS). 4. Calcium hydroxide, distilled water and formocresol. 5. Calcium hydroxide ($Ca(OH)_2$), distilled water and glutaraldehyde. 6. Calcium hydroxide, distilled water and ferric sulfate. All mixed materials were dissolved in medium and diluted to 10, 20, 40 and 80 μl/ml concentrations. A cell colorimetric assay (MTT) was used to detect the viability of L929 cells. Results were compared using one way analysis of variance (ANOVA). Differences in treatment means were analyzed using Student-Newman-Keul's test and were considered significant at $p < 0.05$. The survival rate of treated L929 cell showed statistical differences as the concentrations of the pulpotomy materials increased ($p<0.05$). The highest survival rates were found in groups 3, 5 and 6. Conclusions: It is recommended that low toxicity formulas such as Zinc oxide powder, eugenol and ferric sulfate; Calcium hydroxide, distilled water and glutaraldehyde; or Calcium hydroxide, distilled water and ferric sulfate be used clinically as pulpotomy dressing materials. Further research with randomized clinical trials is needed to verify these clinical success rates.

Introduction

In extensive dental caries management, a tooth is sometimes treated with a pulpotomy. The goals of pulpotomy intervention can be classified as devitalization [formocresol (FC), glutaraldehyde (Glu), electrocoagulation], preservation [ferric sulphate (FeS), calcium hydroxide ($Ca(OH)_2$), mineral trioxide aggregate (MTA), lasers)] and remineralization (indirect pulp therapy, bone morphogenic proteins, collagen) of the dental pulp in a primary molar with extensive caries

(1). Many pulpotomy materials have been clinically applied. There is little data on their toxicity.

Buckley's formocresol was first introduced as a pulp medicament in 1904 (2), and since 1930 (3), it has been the treatment of choice for primary molar vital pulpotomies. A one-fifth dilution of formocresol was as effective as a full strength solution in terms of its initial cytotoxicity on fibroblasts (4,5). Formaldehyde is an ingredient in Buckley's formocresol solution. In June 2004, the International Agency for Research Cancer (IARC) classified formaldehyde as carcinogenic to humans and the dental profession then needed to look for viable alternatives to formocresol (6). Glutaraldehyde was proposed as a new pulp tissue fixative by -Gravenmade in 1975 (7) and has been reported to be a better tissue fixative than formocresol (8). But its systemic distribution from pulpotomy sites, cytotoxicity (9) and mutagenicity (10) have been reported to be similar to formocresol. It has been used clinically as a replacement for formocresol.

Ferric sulphate (15·5%) has been investigated widely and has been used in animal and human studies as a haemostatic agent in pulpotomy procedures. On contact with blood, a ferric ion protein complex is formed, and the membrane of this complex seals the cut vessels mechanically, producing haemostasis. The agglutinated protein complex forms plugs which occlude the capillary orifices, preventing blood clot formation (11).

Calcium hydroxide has been proposed as an alternative to formocresol for pulpotomies in primary teeth (12). Because fibrous layer and vital pulp tissue are found beyond the calcific bridge (13), calcium hydroxide can be used for either preservation and/or intervention. Thus calcium hydroxide is used in pulpotomies as a base material mixed with formocresol, glutaraldehyde or ferric sulphate.

Because of IARC concerns about formaldehyde carcinogenicity, different medicaments have been selected and applied in pulpotomies. However, cell biocompatibility reports on these materials are lacking, and clinicians are still confused on the best choice of pulpotomy medicaments. Published results on pulpotomy medicaments, included basic and clinical studies, are unclear in

material selection. The purpose of the present study was to evaluate cell toxicity from different pulpotomy materials. It is hoped that this study can provide the clinician with further selection criteria for pulpotomy procedures.

MATERIAL AND METHODS

Material and sample preparation

Six different puplotomy materials were prepared and grouped as follows: 1. Zinc oxide powder 6 g : Eugenol 1 ml: Formocresol 1ml. 2. Zinc oxide powder 6 g : Eugenol 1 ml: Glutaldehyde 1 ml. 3. Zinc oxide powder 6 g: Eugenol 1 ml: Ferric sulfate 1 ml 4. Calcium hydroxide 6 g : distilled water 1 ml: Formocresol 1 ml. 5. Calcium hydroxide 6 g : distilled water 1ml : Glutaldehyde 1 ml. 6. Calcium hydroxide 6 g: distilled water 1 ml: Ferric sulfate 1 ml.

Samples were prepared as follows: freshly mixed materials were placed in glass rings (2 mm in height, 6 mm in diameter) and allowed to set for 24 h at 37 ℃ in a humidified chamber. In the experimental group, five samples of each pulpotomy material were then eluted in 10 ml of cell culture medium at 37 ℃, in air and 5% CO_2 for 24 hours. After that, the materials were centrifuged at 10000rpm for 10 minutes. The supernatant were used to prepare different concentrations of the test materials. The concentrations of the test materials were diluted by adding culture medium to final concentrations of 10, 20, 40 and 80 μl/ml. The pure culture medium without any experimental material served as the control group.

Cell viability test by MTT((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide colorimetric) assay

The assay procedure was the same as described in our previous study (14). The procedure was as follows: a mouse cell fibroblast cell line (L929) was routinely cultivated in DMEM medium (Sigma Chemical, St. Louis, Mo, USA) supplemented with 5% fetal bovine serum (Sigma Chemical, St. Louis, Mo, USA) at 37 ℃ in air and 5% CO_2. Single-cell suspensions of L929 cells were obtained from monolayer cell cultures close to confluency after trypsinization. Cell numbers were

determined by hemocytometric counting, and 10^4 cells/well were seeded into 96-well plates. Cells were then incubated for 24 h in a humidified atmosphere of air and 5% CO_2 at 37 °C. Cell cultures were exposed to 10, 20, 40 and 80µl/m concentrations of the experimental materials. Exposure of cell cultures was stopped by discarding the exposure medium after 24 h. Viable cells in both treated and untreated cell cultures were stained with formazan dye MTT (1 mg/ml) (Sigma Chemical, St. Louis, Mo, USA) dissolved in a 200 µl culture medium. After 3 h at 37 °C, the MTT solution was discarded and formazan crystals were solubilized with 200 µl of DMSO. All experiments were performed in triplicate. Optical densities were measured at 570 nm in a multi-well spectrophotometer (Hitachi, Tokyo, Japan). The survival rate was calculated as survival % = absorbance of the treated sample / absorbance of the medium x 100%. Results were compared using one way analysis of variance (ANOVA). Differences in treatment means were analyzed using Student-Newman-Keul's test and were considered significant at $p < 0.05$.

Results

The morphology of L929 cells treated with different materials was observed under a microscope at a magnification of 100X (Figure 1). The control group showed normal L929 growth under routine culture (Fig. 1a). The experimental groups which contained formacresol, such as groups 1 and 4, had decreased L929 cell numbers (Fig. 1 b and 1c). Group 2 also had reduced L929 cell numbers (Fig. 1d). The cell growth in groups 3, 5 and 6 was as good as that in the control group (Fig. 1e, 1f and 1g).

The L929 survival rates after treatment with various pulpotomy materials are shown in the table and in Figures 2-7. The survival rates of all test groups showed statistically significant differences as concentrations changed ($p<0.05$). In group 1, at concentrations above 10µl/ml, the L929 survival rates were below 20% (Fig. 2). In group 2, the L929 survival rates showed dose dependent decreases ($p<0.05$) (Fig.3). In group 4, the L929 cell survival rates were all below 20% at concentrations above 10µl/ml (Fig. 4). In groups 5 and 6, the L929 cell survival rates decreased

as the concentrations increased ($p<0.05$) (Fig. 5,6). In group 3, the L929 cell survival rate was severely decreased at a concentration of 80μl/ml (Fig.7).

Discussion

The present results showed low L929 cell survival rates for materials mixed with formocresol (Fig. 2 and 4). This demonstrates that formocresol is toxic to L929 cells. Previous studies showed different results in experiments with formocresol or formaldehyde (15-18). Small amounts of labeled formaldehyde were detected in the liver, kidney, lung and skeletal muscle of dogs after pulpotomy (15). However, another assessment of the systemic distribution and toxicity of formaldehyde following pulpotomy claimed no toxic effects on the liver and kidneys.(16) Similarily, opposite results were found in two studies of allergic effects (17,18). In a study of embryotoxic and teratogenic effects on chick embryos, formocresol showed mutagnic and carcinogenic effects (19). The results of in vitro and animal studies (in vivo) sometimes do not agree. It is proposed that in vitro tests involve direct contact with the cell, but in animal study chemicals are probably diluted by tissue fluid. In addition, some body organs can detoxify these chemicals, thereby reducing damage. Thus the results of cell culture study and animal study can not be compared. Our in vitro study demonstrated that small amounts of formocresol can cause cell death. Clinicians need to be aware of this when they choose materials for a pulpotomy.

Many base materials have been applied in pulpotomy studies, including calcium hydroxide and zinc oxide power. One clinical report on calcium hydroxide dressings and zinc oxide eugenol dressings with glutaraldehyde showed a 73.6% success rate after 12 months follow-up (20). However, in the present study, the two groups with glutaraldehyde showed different survival results. In Figure 3, the survival rate decreased to 50% for the 10μl/ml concentrations in zinc oxide eugenol mixed with glutaraldehyde. But in Figure 5 showing calcium hydroxide mixed with glutaraldehyde, the L929 cell survival rate were shown high survival rate except in the high concentrations 80μl/ml which showed low survival rate. The present in vitro survival result was different from the above mentioned clinical success rate.

It is reported that glutaraldehyde is distributed systemically from the pulpotomy site, and its cytotoxicity and mutagenicity have been reported to be similar to formocresol (21-23). Calcium hydroxide has favourable antibacterial effects, is easily resorbed and causes no foreign body reaction. Zinc oxide eugenol paste dressing has been the material of choice for pulpotomies recently, but concerns have been expressed regarding its rate of resorption. Therefore, calcium hydroxide with glutaraldehyde would be better than zinc oxide eugenol with gluaraldehyde. However one clinical assay showed a high success rate (92.9%) was zinc oxide eugenol mixed with glutaraldehyde (20). Therefore, zinc oxide eugenol mixed with glutaraldehyde can be a good pulpotomy material.

Ferric sulphate (15.5%) has been investigated widely and has been used as a haemostatic agent in pulpotomy procedures in human and animal studies. It is used to improve the efficacy of calcium hydroxide. Failure of calcium hydroxide in one study was attributed to persistent extrapulpal blood clots (24). Ferric sulphate and formocresol have produced equivalent successful clinical and radiographic outcomes (25). Thus, in the present study we selected ferric sulphate mixed with calcium hydroxide and zinc oxide eugenol as test materials. The present study showed high or equivalent survival rates for ferric sulphate mixed with either calcium hydroxide or zinc oxide eugenol for all concentrations (Fig 6 and 7, Table 1). The only exception was for the 80μl/m concentration of zinc oxide eugenol mixed ferric sulphate, which showed a severely decreased survival rate (10.68%) (Fig 7).

It is reported that ferric sulphate appears to be as effective in vital pulpotomies as formocresol, and there is no evidence to date to suggest any adverse effects of this medicament (25). From the present study, the authors propose using ferric sulphate mixed with either calcium hydroxide or zinc oxide eugenol as the treatment of choice for vital pulpotomties.

Conclusions

The results showed high survival rates for a combination of calcium hydroxide or zinc oxide

eugenol mixed with ferric sulphate and for calcium hydroxide mixed with glutaraldehyde. These are suggested to be the best choices in pulpotomy medicament. Further research with long term randomized clinical trials is required to evaluate the success rate.

Acknowledge:

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Legand

Figure 1. The morphology of L929 cells treated with different pulpotomy materials. The magnification is 100x under microscope observation. a. control group. b. ZOE + FC (20 μ l/ml). c.$Ca(OH)_2$ + FC (20 μ l/ml). d. ZOE + Glu (20 μ l/ml).e. $Ca(OH)_2$ + Glu (20 μ l/ml. f. ZOE + FeS (20 μ l/ml). g. $Ca(OH)_2$ + FeS (20 μ l/ml).

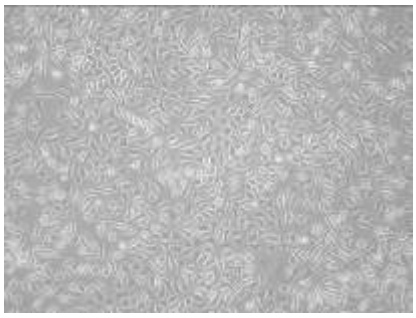
Figure 2. L929 cell survival rate for zinc oxide eugenol mixed with formocresol. Figure 3. L929 cell survival rate for zinc oxide eugenol mixed with glutaraldehyde.

Figure 4. L929 cell survival rate for calcium hydroxide mixed with formocresol. Figure 5. L929 cell survival rate for calcium hydroxide mixed with glutaraldehyde.

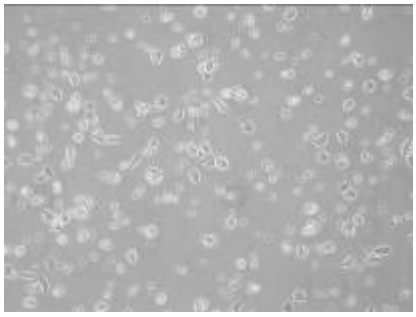
Figure 6. L929 cell survival rate for calcium hydroxide mixed with ferric sulphate.

Figure 7. L929 cell survival rate for zinc oxide eugenol mixed with ferric sulphate.

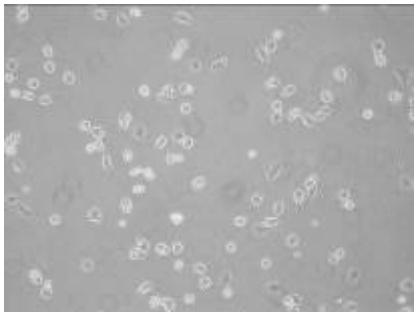
Table I. L929 cell survival rates after treatment with different pulpotomy materials.



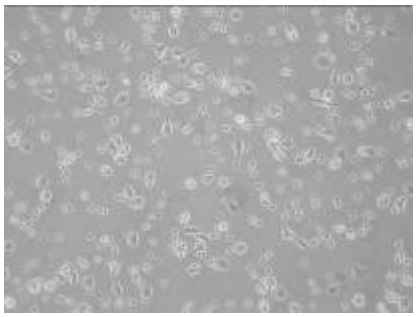
a.Control (100x magnification)



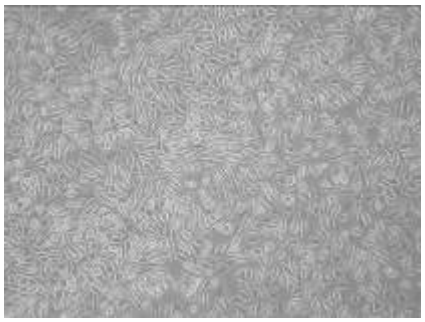
b. ZOE + FC (20 μ l/ml)



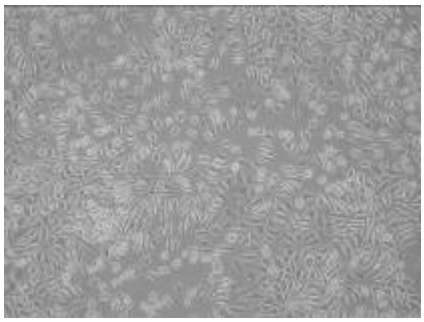
c.$Ca(OH)_2$ + FC (20 μ l/ml)



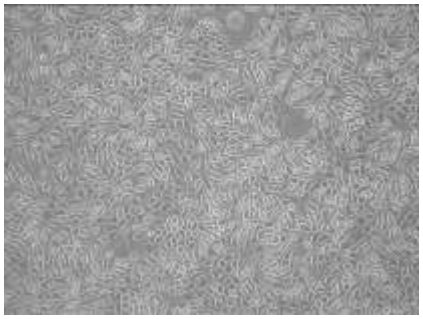
d. ZOE + Glu (20 μ l/ml)



e. $Ca(OH)_2$ + Glu (20 μ l/ml)



f. ZOE + FeS (20 μ l/ml)



g. $Ca(OH)_2$ + FeS (20 μ l/ml)

Figure 1. The morphology of L929 cell treated with different formula pulpotomy materials. The magnificationwas 100x under microscope observation. a. control group. b. ZOE + FC (20 μ l/ml). c.$Ca(OH)_2$ + FC (20 μ l/ml). d. ZOE + Glu (20 μ l/ml).e. $Ca(OH)_2$ + Glu (20 μ l/ml. f. ZOE + FeS (20 μ l/ml). g. $Ca(OH)_2$ + FeS (20 μ l/ml).

Figure 2. L929 cell survival rate for zinc oxide eugenol mixed with

formocresol.

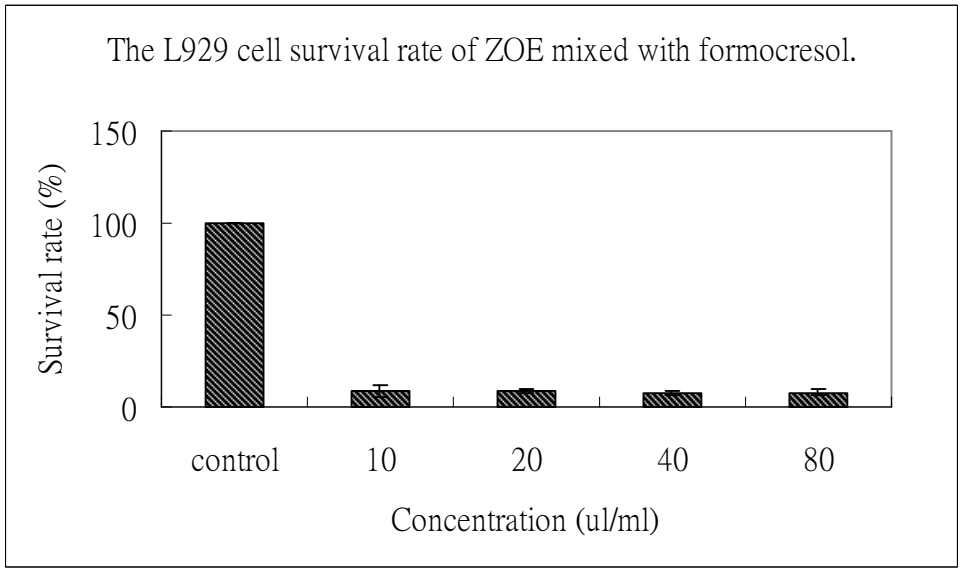


Figure 3. L929 cell survival rate for zinc oxide eugenol mixed with glutaraldehyde.

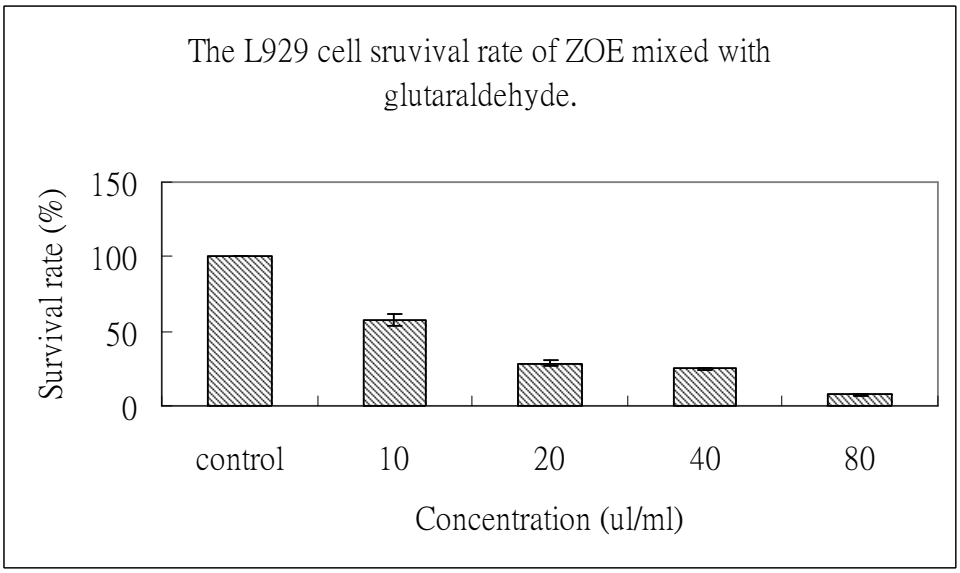


Figure 4. L929 cell survival rate for calcium hydroxide mixed with formocresol

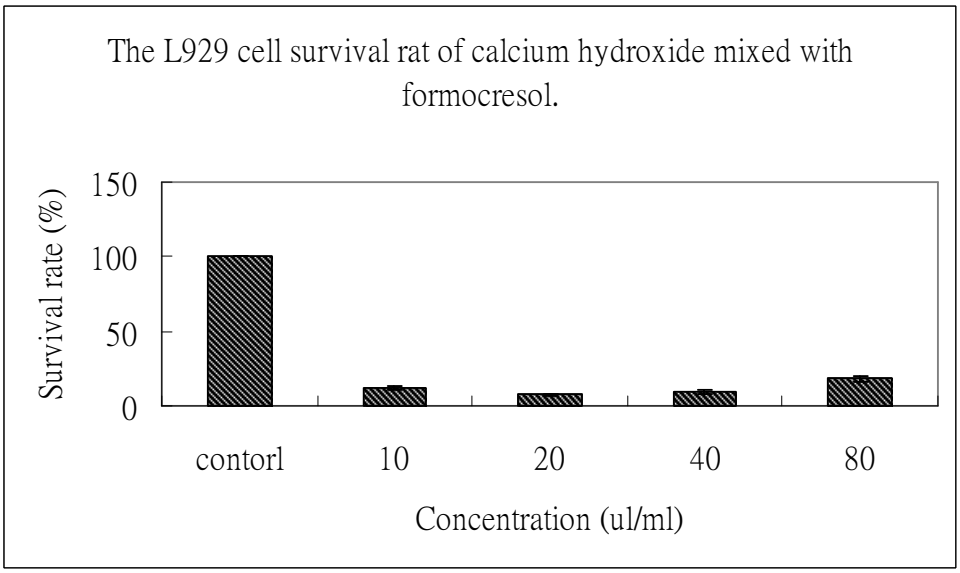


Figure 5. L929 cell survival rate for calcium hydroxide mixed with glutaraldehyde.

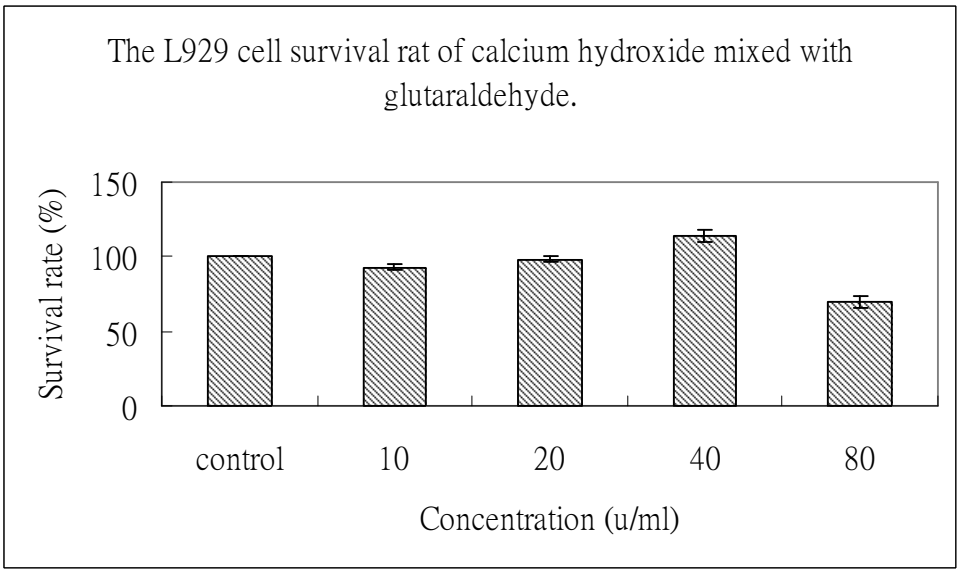


Figure 6. L929 cell survival rate for calcium hydroxide mixed with ferric sulphate.

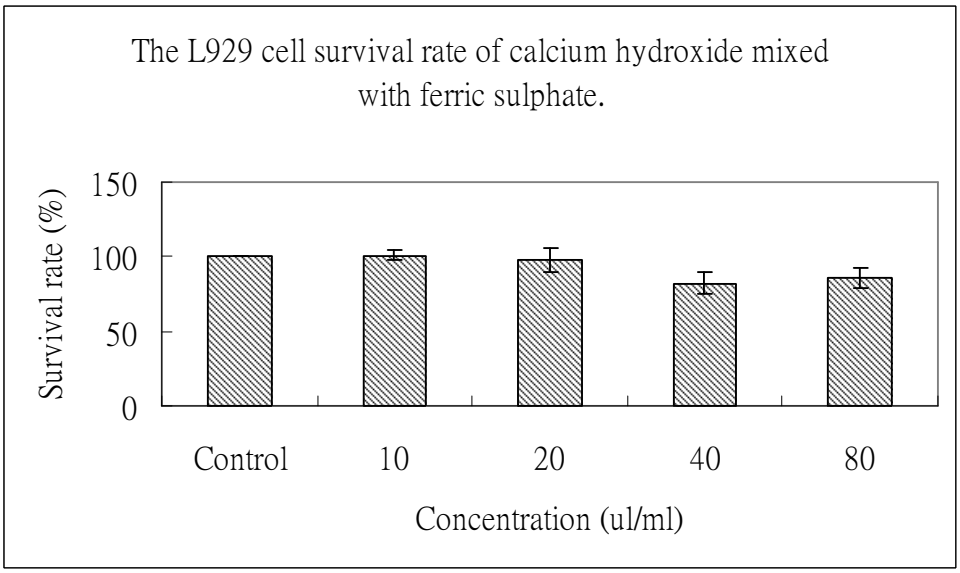


Figure 7. L929 cell survival rate for zinc oxide eugenol mixed with ferric sulphate.

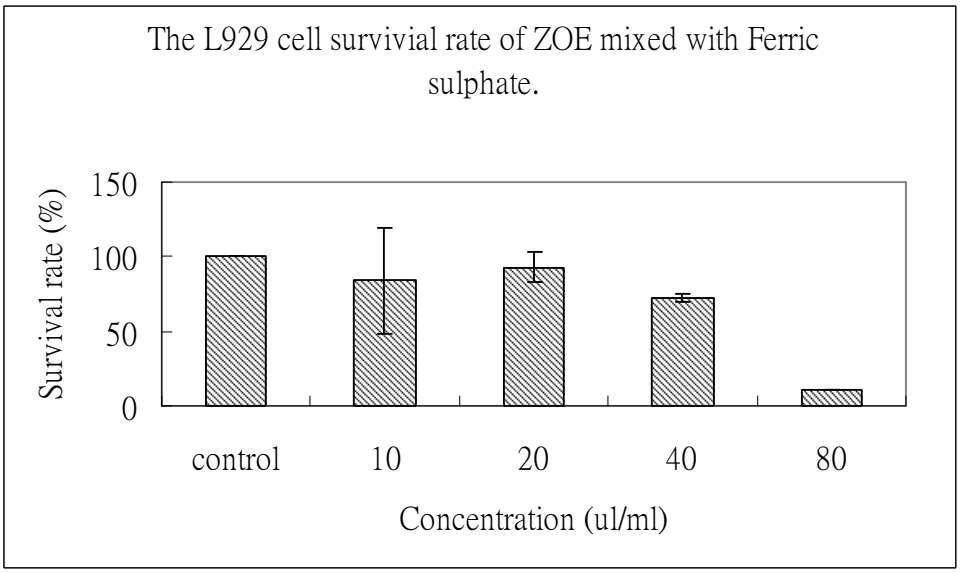


Table I. L929 cell survival rates after treatment with different pulpotomy materials. Aberration: ZOE: zinc oxide eugenol, FC: formocresol, Glu: glutaraldehyde, FeS: ferric sulfate.

		control	10	20	40	80	P<0.05
ZOE+FC	Mean	100	8.918[a]	8.623[a]	7.63[b]	7.997[b]	Yes
	SD	0	3.294	1.222	1.348	1.298	
ZOE+Glu	Mean	100	57.38	28.52	25.15	7.65	Yes
	SD	0	4.192	1.721	0.7876	0.3397	
ZOE+FeS	Mean	100[a]	84.04[b]	92.68[a]	72.5[b]	10.68	Yes
	SD	0	35.81	10.27	2.381	0.6246	
$Ca(OH)_2$+FC	Mean	100	12.54	7.778[a]	9.082[a]	18.35	Yes
	SD	0	1.339	0.8984	0.9753	1.78	
$Ca(OH)_2$+Glu	Mean	100[a]	92.46	98.29[a]	113.8	69.38	Yes
	SD	0	1.989	2.369	4.465	4.189	
$Ca(OH)_2$+FeS	Mean	100[a]	101.1[a]	97.42[a]	82.24[b]	85.41[b]	Yes
	SD	0	3.819	8.226	7.541	6.762	

One-way ANOVA was used to test for significant differences among different concentrations. The Student-Newman-Keul (SNK) multiple comparison of means procedure at $P < 0.05$ was used to show differences. SNK ranking with the same letters do not significantly differ at $P = 0.05$.