行政院國家科學委員會專題研究計畫 成果報告

矽鈣合成物於體外與體內之生物活性研究(第 3 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-040-021-MY3
執 行 期 間 ：99 年 08 月 01 日至 100 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：黃翠賢
共同主持人：高嘉澤
計畫參與人員：學士級-專任助理人員：謝婉玉

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 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 100 年 09 月 13 日

行政院國家科學委員會補助專題研究計畫

成果報告

（矽鈣合成物於體外與體內之生物活性研究）

計畫類別：□個別型計畫　□整合型計畫
計畫編號：NSC 97－2314 －B －040 － 021 －MY3
執行期間：97 年 08 月 01 日至 100 年 07 月 31 日

執行機構及系所：中山學大學牙醫系

計畫主持人：黃翠賢
共同主持人：高嘉澤
計畫參與人員：謝婉玉

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

本計畫除繳交成果報告外，另須繳交以下出國心得報告：
□赴國外出差或研習心得報告
□赴大陸地區出差或研習心得報告
□出席國際學術會議心得報告
□國際合作研究計畫國外研究報告

處理方式：除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

中　華　民　國 100 年 09 月 10 日

目錄

中文摘要 --3
英文摘要--4
前言 -- 5
目的 -- -5
文獻探討 --5
研究材料與方法 --7
結果與討論 -- 9
結果與建議 --11
參考文獻 --12
圖表 --15
成果報告自評 -- 29

中文摘要

關鍵字：鈣矽類材料、細胞相容性、西方膜點分析、動物試驗、發炎反應、骨細胞傳導效應

將牙根管通道系統及其表面可能的通道封閉是逆向充填或是牙根修復材料最為主要的目的。因此良好的生物相容性也是必備的要素，除了不會造成細胞毒性之外，最好能具有引導新骨再生、牙週組織附著、牙骨質再生的能力。

因此本研究目的：

1. 於體外試驗，探討自行合成之MTA之對細胞發炎免疫方面反應。
2. 於體外試驗，探討自行合成之MTA於細胞內對於骨生成因子之訊息變化。
3. 於體內試驗，探討自行合成之MTA於動物體內植入後之生物體反應。

研究材料與方法：

第一與第二年為體外試驗。依過去的製程MTA燒結出，配製成所需的溶液，細胞採用U2OS 細胞株。於細胞發炎ELISA分析，以材料作用於細胞後，分析有關於負責活化骨細胞之細胞素如； IL 1α,1β和IL6之變化。同時也分析osteocalcin，alkalaine phosphatase之活性。以鹼性磷酸脢(alkaline phosphatase [ALP] activity)分析材料與細胞作用後之活性。以RTPCR方法，觀測細胞COX-2的表現。以西方墨點分析探討細胞內訊息蛋白COX-2 protein、ERKinase、 JNK kinase、caspase 3 、alkaline phosphatase, osteonidogen, osteonectin, 和 osteopontin之的表現，以了解自行研發材料的生物活性機轉。第三年為作用於老鼠體內的試驗，以60隻Sprague Dawley rat作為植入之對象，以合成之MTA為實驗組，另外以Hydroxy apatite 作為控制組，作為比較組織反應之差異性。植入部位為parietal bone和subcutaneous位置，觀察記錄其組織之發炎反應，並紀錄之。所有結果居已統計軟體分析比較結果。

研究結果顯示

CS材料對於U2OS細胞具有良好之生物相容性，隨著濃度與作用時間改變，其細胞生長現象良好。在發炎因子COX-2表現上，CS與MTA一起比較顯示於第三天有明顯COX-2表現，第七天則無差異。 Interleukin 的表現方面，IL1α，IL1β和IL6的表現，CS和MTA的表現無差異，但比控制組的表現高。CS材料對於MG63細胞具有良好之生物相容性(Fgure 6.)，隨著濃度與作用時間改變，其細胞生長現象良好。MTA與CS材料之ERKinase 表現較控制組佳，代表有良好之細胞增殖反應；對於骨誘導生成因子ALP, Type I collegenase, osteocalcin, Bone sialoprotein 和 osteopontin之表現也都呈現明顯反應。不論是MTA或是CS材料於動物體內植入第六週均有呈現發炎反應，到第十二周，發炎效應就降低。組織埋入與切片結果顯示材料對於生物體據有良好生物相容性。

結語：

本計劃顯示本研究室自行合成之鈣矽類材料據有良好之生物相容性，日後可以應用於臨床上。

Abstract

Key words:

Calcium silicate cement, biocompatible western blot assay, animal study, inflammation , osteoconduction

The root end filling material is used on the root apex or pulp perforation repair. The material will contact with tooth and surround tissue. It is needed material have good biocompatibility. The ideal material should have good physical properties, biocompatibility and may have osteoconduction or tissue regeneration ability. The purpose of present project was to 1. Investigate the calcium silicate (CS) and MTA inflammation or immune reaction. 2. The CS and MTA osteoconduction ability. 3. The material implantation reaction on rate. Material and methods: Follow the previous study, CS were fabricated from our laboratory. The CS material after preparation were compared with MTA. The control group in present were blank group. The MTT assay were to study the cell survival rate. The western blot assay and RTPCR assay were used to identify following marker expression. They were included: COX-2 protein、ERKinase、JNK kinase、caspase 3 、alkaline phosphatase, osteonidogen, osteonectin, osteopontin proteins. The material after preparation were implanted into rat submucosa and parietal bone. The HE stain were to evaluate the inflammation reaction. The data were all statistically analysed to compare the difference. The result showed as follows: 1. The CS and MTA were all biocompatible with U2OS cells. It can promote the cell growth. The ERK Kinase showed increasing expression . 2. The inflammatory marker COX2 were appeared when initial contact with cells. And were decreasing as culture time increased. It represent no inflammation reaction happened. 3.The expression of IL1α，IL1β, IL6 showed MTA and CS were have different degrees changes. The outcome showed materials were no immune reaction to cell. 4. The MG63 bone cell were also biocompatible with CS and MTA. The bone marker expression were obviously appeared on ALP, Type I collegenase, osteocalcin, Bone sialoprotein ,osteopontin proteins. 5. The implantation test on rat, the subcutaneous tissue showed mild inflammation reaction, not in parietal bone implantation. No dominant bone formation were found in bone tissue.

Conclusion: The CS material were biocompatible with cell in vitro study and in animal in vivo study. This material can be used in clinical after further clinical study test.

前言

績前次計畫，本研究室已可以合成鈣矽類材料，目前市面上有的根尖充填材料以三氧礦聚合物(MTA)為主，其產品發表至今以超過十年以上，由於治療上效果佳因此廣為應用。由於其費用昂貴，且有其他缺點，因此引起本研究計畫之動機，嘗試由自己合成之物質後分析比較生物相容性，最終期望能改進材料之缺點、商品化，並降低其費用，以利醫師與患者之使用。

研究目的

本研究以三年時間完成自行合成MTA之一系列體外與體內之生物相容性研究，因此研究目的包括：

一、 於體外試驗，探討自行合成之MTA之對細胞發炎免疫方面反應。

二、 於體外試驗，探討自行合成之MTA於細胞內對於骨生成因子之訊息變化，以了解材料對於細胞之活性機轉。

三、 於體內試驗，探討自行合成之MTA於體內植入後之生物體反應。

文獻探討

茲就材料之相關文獻作介紹如下：

1. 三氧礦聚合物(MTA)成分

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。[1] 根據材料廠商之敘述，MTA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)。傳統上MTA應用於根管治療上較多，但由下面之研究發現，MTA對骨頭之生成似乎也扮演著某種角色。

2. MTA生物相容性－細胞影響

關於 MTA 之生物相容性研究常見有許多方式，有觀察細胞之表面與生長，皮下植入或是骨頭內植入研究或是直接與牙周組織接觸等方式。於細胞學之細胞毒性研究方面，大多數學者認為MTA具有細胞相容性，[2-8] 其中 之細胞毒性測試方式不盡相同， 如Keiser et al.(2000), Huang et al.(2003),Camilleri et al. (2004)[9]等應用methyltetrazolium (MTT) 方法分析細胞之細胞毒性， Torabinejad et al.(1995) 則應用agar overlay 方式與rediochromium 釋放之方法分析細胞毒性。[8] 而Haglund et al(2003) 以SEM觀察分析細胞之存活，則認為MTA對於macrophage 和fibroblast 具有細胞毒性。[10] 但由臨床上之使用，尚未見使用MTA後有任何不適之病例報告出現，因此大多研究認為MTA材料是可以被生物體接受之材料。

3.MTA之發炎效應

另外MTA有關於細胞素反應之研究，主要以探討白介素(interleukin)之表現居多，用於觀測細胞之分化(differentiation)現象。MTA會誘導骨細胞之inflammatory cytokine表現，與良好的細胞附著效應。過去本研究室也針對商用MTA做過探討，發表之研究結果(Huang et al. 2005) 顯示MTA會促使骨細胞(U2OS)之IL4和IL10分泌增加，[11] 在Loma Linda大學，Mitchell et al.研究則發現MTA作用細胞後IL-6, IL-8會增加，而IL-1α與IL-1β不會有增加。[12] 相反的，Koh et al.發現IL-6，IL-1α與IL-1β量於MTA材料與細胞接觸後6天後會增加。[7, 13] 即發炎現象會出現。

發炎之反應，PGE2為發炎之介質，當組織受到外來之刺激如化學藥物之刺激或是細菌之刺激，宿主體內會有發炎性之反應出現。 [14-16] Cycloxygenase (COX)為prostaglandin endoperoxide synthase，它負責prostaglandin 之生物合成。COX屬於dual enzyme，它可催化二種enzyme的反應：1.藉由cycloxygenase 將arachidonic acid轉變為PG2。2.藉由peroxidase減少PG2變為PGH2。基本上COX可分為constitute enzyme(COX1)和inducible isoenzyme (COX2)，其中COX2可因Proinflammatory 因子而將之誘導出來。過去有關MTA於此發炎方面之探討未見有報告，本計劃將以RT-PCR方式，探討比較商品化之MTA與自行合成MTA之細胞發炎COX-2表現。

4. MTA之細胞內訊息因子變化

Bonson et al.則發現MTA會誘使牙周韌帶細胞與纖維母細胞之alkalain phosphatase 表現並增加其活性。[17] 過去本研究室(2003)已發表 MTA對U2OS骨細胞之具生物相容性，對於U2OS細胞受MTA釋出物刺激後，其細胞內訊號傳遞訊號 Erk kinase 會有明顯之表現增加，此研究中ERK 出現代表U2OS骨細胞會有增生(proliferation)之能力。[6] 基於此動機，本研究計畫將探討目前本研究室開發出之MTA材料對細胞作用後， alkaline phosphatase, osteonidogen, osteonectin, 和 osteopontin等指標之反應。

5. MTA生物相容性－活體植入

過去學者(Moretton et al. 2000, Yaltirik et al. 2004) 有將MTA植入到皮下觀察其反應，結果發現有coagulation necrosis和dystrophic calcification出現，反應隨時間增加而減緩。[18,19] 過去Kao et al.(2006)發表將商品化之MTA植入老鼠體內觀察其組織反應，結果與上述作者研究結果相似。[20] Moretton et al.研究指出於植入試驗中觀察到有osteogenesis出現。Saidon et al. (2003) 於guinea pigs 植入研究中發現骨頭有癒合現象與少量發炎反應。[21] Loma Linda大學研究人員將MTA植入tibia和下顎骨後其組織相容性很好，沒有發炎反應出現，於tibia中有骨頭沉積(bone deposition)出現。[1,22]

商品化MTA於牙根周圍組織之反應研究結果發現它可誘導硬組織之生成，牙周組織再生。[23] Economides et al.(2003)發現MTA早期癒合反應之出現，乃組織先出現硬組織之生成，然後沿著軟組織與MTA交接面繼續形成。[24] 新調製或是放置一段時間之MTA，都可產生新的cementum。[25] 基於此動機，本研究計畫將以自行合成之改質MTA做植入動物試驗，觀察是否組織會發炎與骨化之反應出現。

材料與研究方法

一、MTA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。

由自行合成之MTA材料將之製成直徑為3mm高度2mm之圓柱體，將之浸泡於細胞培養液中，其中將MTA調製後分為調製一小時與硬化後二十四小時組，各組分別浸泡二十四小時與七天，之後經離心取其上層液做下面之測試。

本研究計畫將以Mineral trioxide aggregate (MTA)[包括自行合成與商品化之白色MTA]、calcium hydroxide 、與Hydroxyapatite 三大類材料作為研究材料。根據Sarker等(2005, vol 33 J Endod) 研究發現MTA於體液中接觸後會形成Hydroxyapatite之成分而有助於骨之生長。因此本年度將探討材料作用後細胞是否有發炎反應之出現，再者研究其是否有讓細胞之成骨標記出現。

二、自行合成MTA於細胞內對於骨生成因子之訊息變化

二. 1. U2OS細胞培養

細胞株培養於McCoy’s medium，內含有10% feta bovine serum, 100ug/ml streptomycin, 100 ug/ml penicillin and 0.25 ug/ml amphotericin.。於細胞培養中與合成材料一起培養1,6,12,24,48,72, 144 and 192 hours.，之後以carbon coating後，上面放蓋玻片觀察。

二.2 細胞發炎之分析

分析當材料作用於細胞後，有關於負責活化骨細胞之細胞素如； IL 1α,1β和IL6之變化。同時也分析osteocalcin，alkalaine phosphatase 之活性。

此部分方法參考Koh et al.之實驗步驟；[26]

1. 細胞培養中與合成材料一起培養1,6,12,24,48,72, 144 and 192 hours後，去除培養液，放置於-20　中，直到開始作ELISA試驗。
2. 以protein washing buffer清洗，加入100ml 1% BSA於 37　下置於水中反映一小時。
3. 將1ug/ml mouse anti human IL 各取50ul 置於培養盤中，於37　下作用，加入10ug/ml goat antimouse conjugated AP 50ul於培養盤中。
4. 與mouse anti human IL作用1 小時，以protein buffer 洗三次
5. 加入NBT/BCIP (100ul/well)於避光室溫下作用30分鐘
6. 放於ELISA reader 570nm內讀取。

二.3. 鹼性磷酸脢(alkaline phosphatase [ALP] activity)分析

將參照Sigma公司之建議方法操作:

1. 將處理過之細胞裂解。。
2. 細胞以phosphate buffered saline 洗三次，
3. 利用加入2-amino-2 methyl-1-propanol buffer培養 15 min (Sigma, Procedure 104).
4. 以10ml 0.05% NaOH作為反應中止溶液，於400-420nm.波長下測p-nitrophenol

***三，自行合成MTA對細胞發炎免疫方面反應觀察* COX-2 expression --- reverse transcriptase polymerase chain reaction(RT-PCR 分析)]**

將MTA 、calcium hydroxide 、與Hydroxyapatite作用於細胞後，於1, 3, 6小時分別收取RNA，進行下述之實驗觀察其發炎反應。

實驗步驟參考Joki et al. 與 Nathan et al.方法[27,28]

1. 利用TRIzol (Molecular Research Center Inc., Cincinnati, OH)製備RNA
2. 於15 µl 含有100 mg random hexamer和200 units of Moloney murine leukemia virus reverse transcriptase中，，由RNA製備單股DNA。
3. 以20 µl水稀釋反應混合物，取其中之3 µl作為polymerase chain reaction (PCR).。
4. PCR reaction mixture 含有10 pmol 正向與反向引子和2 units的 Tag DNA polymerase.
5. 熱循環放大次數以25週期作為GAPDH之反應，以35週期作為其他receptor之反應。
6. 每一週期條件設定為於 94°C 1 min 的 denaturation, 於57°C 1 min的annealing 和於72°C 1min of extension.
7. 引子序列設計如下：

a) GAPDH Forward: 5'-TCCTCTGACTTCAACAGCGACACC-3'
   Reverse: 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3'
b) COX-2 Forward: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'
   Reverse: 5'-AGATCATCTCTGCCTGAGTATCTTT-3'

8. PCR的產物以 agarose gel electrophoresis.分析。
9. 強度以densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA)分析記錄.

四、MAPK kinase analysis- 分析細胞接受MTA刺激後，胞內訊息蛋白之表現。

利用西方墨點分析法，將MTA 、calcium hydroxide 、與Hydroxyapatite作用於細胞後，觀察細胞中COX-2 protein、ERKinase、alkaline phosphatase, osteonidogen, osteonectin, 和osteopontin之表現。

試驗方法參考本實驗室與其他作者之方法做Western blot analysis，齊步驟如下 29-30]

1. 處理過之U2OS cells以冷的 PBS洗一次。
2. 1x105細胞於lysis buffer (1% Triton X-100, 0.5% NP40, 10 mM EGTA, 0.2 mM Na3VO4, 0.2 mM NaF, and 0.2 mM PMSF). 中裂解。
3. 將上述做離心[條件 15,000 x rpm for 15 min at 4 ^{0}C.]。25ug蛋白於1 x SDS gel-loading buffer (125 mM Tris, pH 6.8, 5% glycerol, 28 mM SDS, 1% beta-mercaptoethanol, and 0.006% brompophenol blue)煮沸5 分鐘。
4. 蛋白以12.5% SDS-PAGE分離，並轉移至polyvinylidend difluoride membranes.上，於室溫下處理1小時 [條件為3% BSA, 5% nonfat dried milk, 10 mM Tris, pH 7.5, 100 mM NaCl, 和0.1% Tween 20]
5. 以TBS-T buffer洗四次，membrane浸於含有0.5 µg/ml rabbit antibody過夜。

6. 再以 second antibody處理一小時，之後以TBS-T buffer 洗 20 min.
7. 結果以densitometer and the SCION image program處理。 [29]

五、動物逐入試驗

將植入subcutaneous之材料分成二類，一類製成2mm厚 5mm直徑之disc。另一類則新鮮混合後將材料加入空針中，在直接注入皮下組織(subcutaneous)。植入Parietal bone則將材料混合後直接植入骨中，分別於第一週，第六週與第十二週犧牲老鼠。

實驗手術部分參考過去本研究室動物試驗模式，此部分已通過動物及人體試驗委員會之審查。步驟進行方式如下；

1. 老鼠以 Ketamine.(0.14 mL/100 g body weight)做肌肉下注射
2. 於麻醉下，將老鼠的頭頸背的毛剔除，以碘酒消毒
3. 於mid-dorsal 和 pelvic dorsal部位做一10mm長的切口，以提供做材料植入。
4. 另外於Pouches處也做切線，大小也是10mm長的切口，以4-0 silk sutures作縫合。
5. 於眼睛後方做一相對切線以作為材料在intraosseous 植入，將頭皮翻開到頂骨後，用高速手機#6 round bur做打洞，此時應避免穿過dura mater層。
6. 以生理食鹽水做為冷卻，開口大小約1.5mm直徑大。
7. 再將植入物植，以4-0 silk sutures作縫合。
8. 動物於手術後嗣天內，每日接受A Flo-cillin (0.1 mL) (The Fort Dodge Co., Fort Dodge, IA) 注射，另外皮下注射則用 Torbugesic (0.03 mL) 每日二次共計五天。
9. 分別於第7 days, 6 weeks 和 12 weeks將動物犧牲，犧牲前二日給予染料餵食。
10. 做組織切片，以下述方法比較反應。

實驗結果之觀察比較

[參考Moretton et al. 提出之方法 31, 32]，

將皮下組織(superior, inferior, and two lateral sides)發炎反應做等級之分類，分別為：

0 = none (no detectable inflammation);
1 = mild (scattered leukocytes);
2 = moderate (localized dense leukocytic infiltration);
3 = severe (diffuse dense leukocytic infiltration).

將骨織發炎反應依照epicranial surface部位(the endocranial and lateral surfaces of the implant are graded)做等級之分類，分別為：

0 = none (no detectable inflammation);
1 = mild (scattered leukocytes);
2 = moderate (localized dense leukocytic infiltration);
3 = severe (diffuse dense leukocytic infiltration)

The grades of the four surfaces are then averaged and that average score used as the final score for that implant site.

等級分類標準如下依據；

0 = none (no evidence of osteogenesis);
1 = slight (occasional islands of osteogenesis over the respective surface);
2 = moderate (at least half of the respective surface partially covered by bone);
3 = extensive (complete coverage or “bridging” of the respective surface with bone)

統計分析

統計法比較各組差異性。

結果與討論（含結論與建議)

結果一、

CS材料對於U2OS細胞具有良好之生物相容性(Figure 1)，隨著濃度與作用時間改變，其細胞生長現象良好。

討論一

過去XRD研究顯示，CS材料之結晶結構與MTA之結構相仿，MTA 於許多文獻上顯示對於不同來源細胞均據有優良之生物相容性，本研究以CS為對象，對於骨細胞株同樣據有相同生物相容性。本研究結果之濃度隨著時間增加呈現齣戲胞生長遞增之現象。

結果二

在發炎因子COX-2表現上，CS與MTA一起比較顯示於第三天有明顯COX-2表現，第七天則無差異。(Figure 2)

討論二

一般細胞對於外來之材料反應不外是排斥或是接受，因此當材料與系胞接觸後，細胞為了自我防衛，會刺激細胞一系列之保護機制，其中發炎即為其中一種模式。COX2之表現顯示細胞有勁入發炎狀態，但是經過第三天後，其反應之表現明顯下降，代表一開始可能是保護作用，接續細胞無壞死或破壞，因此細胞開始接受材料。相同的於商品化之MTA 也產生類似之發炎效應。

結果三

Interleukin 的表現方面，IL1α，IL1β和IL6的表現，CS和MTA的表現無差異，但比控制組的表現高。(Figure 3-5）

討論三

由 COX 2之表現得知細胞有出現發炎，因此探討細胞免疫機制上之蛋白反應，我們選擇觀察第三天 IL1α，IL1β和IL6蛋白之表現，發現CS 之 IL1α表現略高於MTA 之 IL1α表現； 發現CS 之 IL1β表現略低於MTA 之 IL1β表現；而IL6蛋白之表現則於MTA與CS表現尚無差異性。然而此結果與控制組比較，發現實驗組均有比控制組高的蛋白表現。MTA於早期研究也顯示對於骨細胞之研究上，也呈現不同之IL2, 6, 8 之蛋白表現。

結果四

CS材料對於MG63細胞具有良好之生物相容性(Fgure 6.)，隨著濃度與作用時間改變，其細胞生長現象良好。

MTA與CS材料之ERKinase 表現較控制組佳，代表有良好之細胞增殖反應；對於骨誘導生成因子ALP, Type I collegenase, osteocalcin, Bone sialoprotein 和 osteopontin之表現也都呈現明顯反應 （Figure 7.)

討論四

由於U2OS細胞株細胞表現不穩定，因此採用同為骨細胞株之MGF63作為接續研究。相同的，CS 與MTA作用於MG63細胞以MTT觀察其細胞之生長，結果顯示細胞之生長良好，二種材料不具有統計學上差異。結果與材料作用於U2OS細胞相仿。

於西方墨點分析中，MTA 與CS對於MAPK kinase 之 ERKinase表現顯示均有讓細胞增殖之反應，相對於控制組有明顯增加。此即材料對於細胞生長有正向之反應。

由於材料應用充填於根尖時，一端為牙齒，另一端為接觸骨頭，因此材料對於骨頭之反應

為何，也是值得研究，因此將材料作用於骨細胞後，觀察其骨細胞之生成反應為何，以RTPCR試驗ALP, Type I collegenase, osteocalcin, Bone sialoprotein 和 osteopontin之表現，結果顯示二種材料，對於這些蛋白均有不同時間點反應，代表材料作用後會與骨傳導(Osteocoonduction)之效果，此結果似乎代表這類鈣矽材料具有骨誘導生成，日後也許可以用於某些骨頭之修補。

結果五

植入動物體內之發炎指數如圖8, 9 所示。不論是MTA或是CS材料於第六週均有呈現發炎反應，到第十二周，發炎效應就降低。組織埋入與切片結果顯示於圖10-15.

討論五

如體外之識驗當值入物於短時間之表現顯示，組織有將外來物排出之模式，因此於顯微鏡下比較其發炎反應，隨著植入時間越久，發炎程度有統計上之降低。

植入皮下，發現一有趣之材料反應，CS材料經十二週後，顏色尚無變化，仍呈現乳白色，但是MTA材料則顯示出灰黑色，推測可能是因為組織液與MTA作用，這也代表MTA與CS材料可能結晶後，其結構上仍有差異，才會導致如此。

於鼠之頭蓋骨植入材料，結構上之變化與軟組織相似，本研究雖無計畫觀察骨頭之生成，然於此部份之觀察上，以VonKosa 染色後，似乎差異性不大。於腿骨周邊部份之材料植入，以X光觀察顯示，材料有被纖維組織包裹，此也視為正常之組織反應。

結論與建議

總觀本研究計畫發現自己實驗室合成之鈣矽類材料，其基本物理性質、化學性質與物生相容性於體外試驗均表現室可以接受的材料，與商品化之MTA相比更顯示出其CS性質上相近，且有更優良之表現，例如不會變色。於動物體內試驗，結果顯示不會造成動物之傷害甚至致死。因此，本研究室認為此材料可以作為臨床上之使用，而不會對人體產上不良反應。接續有機會再進行的工作，將申請IRB人體試驗，如果許可情形下，將可更確立此材料之應用。

建議：

由於材料之合成後，許多研究人員對於此材料日後能否上市使用之知識不足，往往流於學術上之發表而已，因此建議可以多提供這方面之相關知識給研究人員。

Reference

1. Torabinejad M, Hong Cu, McDonald F, Pitt Ford TR. Physical and chemical properties of a new root end filling material. J Endodont 1995;21:349-353
2. Asrari M, Lobner D (2003) In vitro neurotoxic evaluation ofroot-end-filling materials. Journal of Endodontics 29, 743–6.
3. Baek SH, Plenk H, Kim S (2005) Periapical tissue responses andcementum regeneration with amalgam, Super EBA, andMTAas root-end filling materials. Journal of Endodontics 31, 444–9.
4. Balto HA (2004) Attachment and morphological behavior of human periodontal ligament fibroblasts to mineral trioxide aggregate: a scanning electron microscope study. Journal of Endodontics 30, 25–9.
5. Hernandez EP, Botero TM, Mantellini MG, McDonald NJ, Nor JE (2005) Effect of ProRoot MTA mixed with chlorhexidine on apoptosis and cell cycle of fibroblasts and macrophages in vitro. International Endodontic Journal38, 137–43
6. Huang TH, Ding SJ, Hsu TC, Kao CT (2003) Effects of mineral trioxide aggregate (MTA) extracts on mitogen-activated protein kinase activity in human osteosarcoma cell line (U2OS). Biomaterials 24, 3909–13.
7. Koh ET, Torabinejad M, Pitt Ford TR, Brady K, McDonald F (1997) Mineral Trioxide Aggregate stimulates a biological response in human osteoblasts. Journal of Biomedical Materials Research 37, 432–9.
8. Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD (1995b) Cytotoxicity of four root- end filling materials. Journal of Endodontics 21, 489–92.
9. Camilleri J, Montesin FE, Papaioannou S, McDonald F, Pitt Ford TR (2004) Biocompatibility of two commercial forms of mineral trioxide aggregate. International Endodontic Journal 37, 699–704.
10. Haglund R, He J, Jarvis J et al. (2003) Effects of root-end filling materials on fibroblasts and macrophages in vitro. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics 95, 739–45.
11. Huang TH, Yang CC, Ding SJ, Yeng M, Kao CT, Chou MY (2005) Inflammatory cytokines reaction elicited by root-end filling materials. Journal of Biomedical Material Research, Part B Applied Biomaterials 73, 123–8.
12. Mitchell PJC, Pitt Ford TR, Torabinejad M, McDonald F (1999) Osteoblast biocompatibility

of mineral trioxide aggregate. Biomaterials 20, 167–73.
13. Koh ET, McDonald F, Pitt Ford TR, Torabinejad M (1998) Cellular response to Mineral Trioxide Aggregate. Journal of Endodontics 24, 543–7.
14. Myers DR, Shoaf HK, Dirksen TR, et al. Distribution of 14Cformaldehyde after pulpotomy with formocresol. Journal of America Dental Association 1978;96:805—13.
15. Block RM, Lewis D, Hirsch J, et al. Systemic distribution of (14C)-labeled paraformaldehyde incorporated within formocresol following pulpotomies in dogs. Journal of Endodontics 1983;9:176—89.
16. Levy S, Noncentini S, Billardon C. Induction of cytogenetic effects in human fibroblast cultures after exposure to formaldehyde or X-rays. Mutation Research; 1983;119: 309—17.
17. Bonson S, Jeansonne BG, Lallier TE (2004) Root-end filling materials alter fibroblast differentiation. Journal of Dental Research 83, 408–13.
18. Moretton TR, Brown CE, Legan JJ, Kafrawy AH (2000)Tissue reactions after subcutaneous and intraosseous implantation of mineral trioxide aggregate and ethoxybenzoic acid cement. Journal of Biomedical Material Research52, 528–33.
19. Yaltirik M, Ozbas H, Bilgic B, Issever H (2004) Reactions ofconnective tissue to mineral trioxide aggregate and amalgam. Journal of Endodontics 30, 95–9.
20. CT Kao, CH Tsai , TH Huang. Tissue and cell reactions to implanted root-end filling materials. Journal of Material Science: Materials in Medicine, 2006 17(9);841-847.
21. Saidon J, He J, Zhu Q, Safavi K, Spangberg LS (2003) Cell and tissue reactions to mineral trioxide aggregate and Portland cement. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics 95, 483–9.
22. Torabinejad M, Ford TR, Abedi HR, Kariyawasam SP, Tang HM (1998) Tissue reaction to implanted root-end filling materials in the tibia and mandible of guinea pigs. Journal of Endodontics 24, 468–71.
23. Regan JD, Gutmann JL, Witherspoon DE (2002) Comparison of Diaket and MTA when used as root-end filling materials to support regeneration of the periradicular tissues. International Endodontic Journal 35, 840–7
24. Economides N, Pantelidou O, Kokkas A, Tziafas D (2003)Short-term periradicular tissue response to mineral trioxide aggregate (MTA) as root-end filling material. International Endodontic Journal 36, 44–8.
25. Ham KA, Witherspoon DE, Gutmann JL, Ravindranath S, GaitTC, Opperman LA (2005) Preliminary evaluation of BMP-2 expression and histological characteristics during apexification with calcium hydroxide and mineral trioxide aggregate. Journal of Endodontics

31, 275–9.

26. Koh ET, McDonald F, Pitt Ford TR, Torabinejad M (1998) Cellular response to Mineral Trioxide Aggregate. Journal of Endodontics 24, 543–7
27. Joki T, Heese O, Nikas DC, Bello L, Zhang J, Kraeft SK, Seyfried NT, Abe T, Chen LB, Carroll RS, Black PM Expression of cyclooxygenase 2 (COX-2) in human glioma and in vitro inhibition by a specific COX-2 inhibitor, NS-398 Cancer Res. 2000 Sep 1;60(17):4926-31
28. Nathan CA, Leskov IL, Lin M, Abreo FW, Shi R, Hartman GH, Glass J. COX-2 expression in dysplasia of the head and neck: correlation with elF4E. Cancer. 2001 Oct 1;92(7):1888-95
29. Volm M, van Kaick G, Mattern J. Analysis of c-fos, c-jun, c-erbB1, c-erbB2 and c-myc in primary lung carcinomas and their lymph node metastases. Clin Exp Metastasis. 1994 Jul;12(4):329-34.
30. Tsui-Hsien Huang, Shinn-Jyh Ding, Ting-Cheng Hsu and **Chia-Tze Kao** Effects of mineral trioxide aggregate (MTA) extracts on mitogen-activated protein kinase activity in human osteosarcoma cell line (U2OS) Biomaterial 2003, 24: 3909-3913
31. Wataha JC, Craig RG, Hanks CT. Precision and new methods for testing in vitro alloy cytotoxicity. Dent Mater 1992;8:65-71
32. Moretton TR, Brown CE, Legan JJ, Kafrawy AH (2000)Tissue reactions after subcutaneous and intraosseous implantation of mineral trioxide aggregate and ethoxybenzoic acid cement. Journal of Biomedical Material Research52, 528–33.

Figure 1. The Calcium silicate (CS) material treated on U2OS cell viability test by MTT assay.

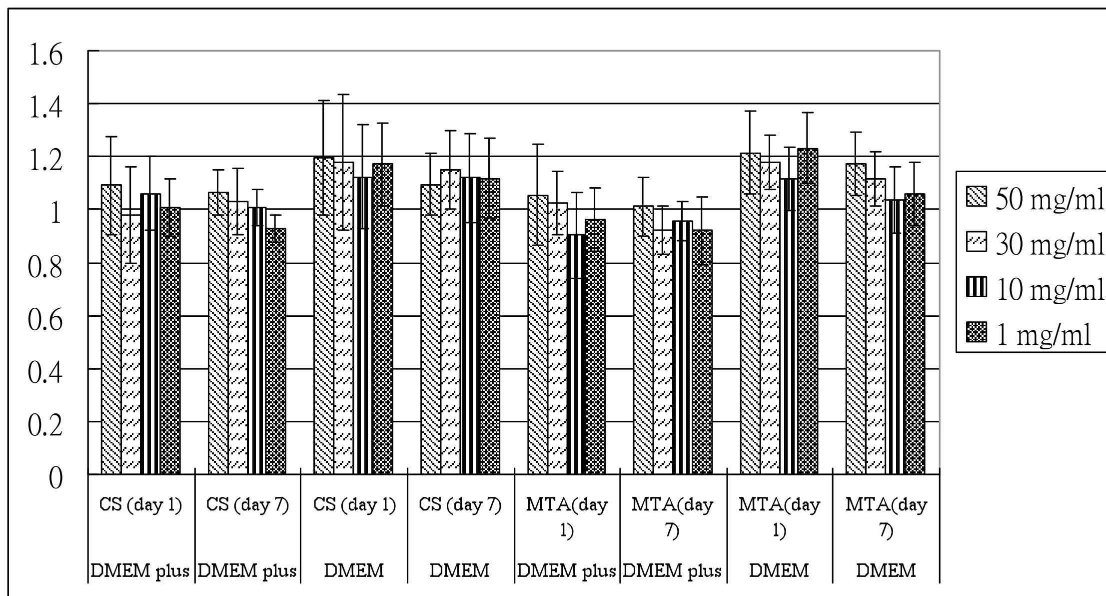


Figure 2. The COX -2 expression comparison of CS material and MTA.

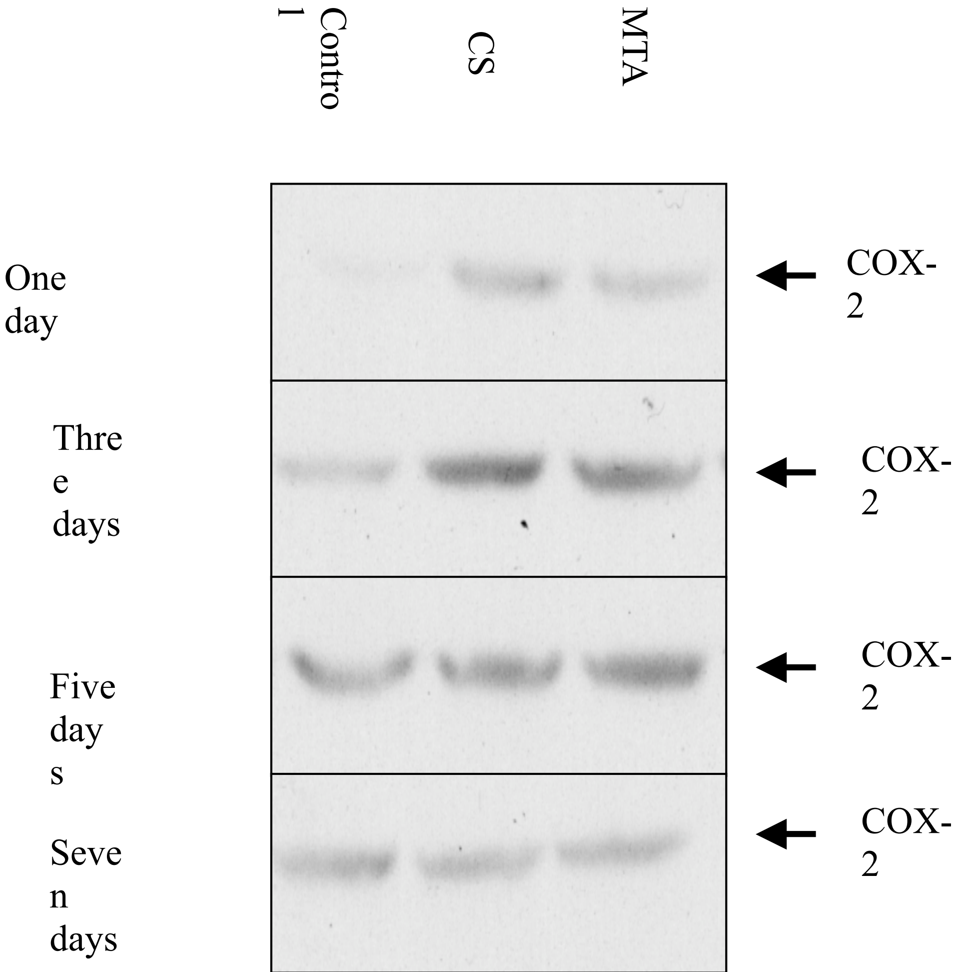


Figure 3. Mean expression of IL-1 alpha from U2OS cells in the presence of different materials at 24 h. The sample size is four. Bar ± standard deviation.

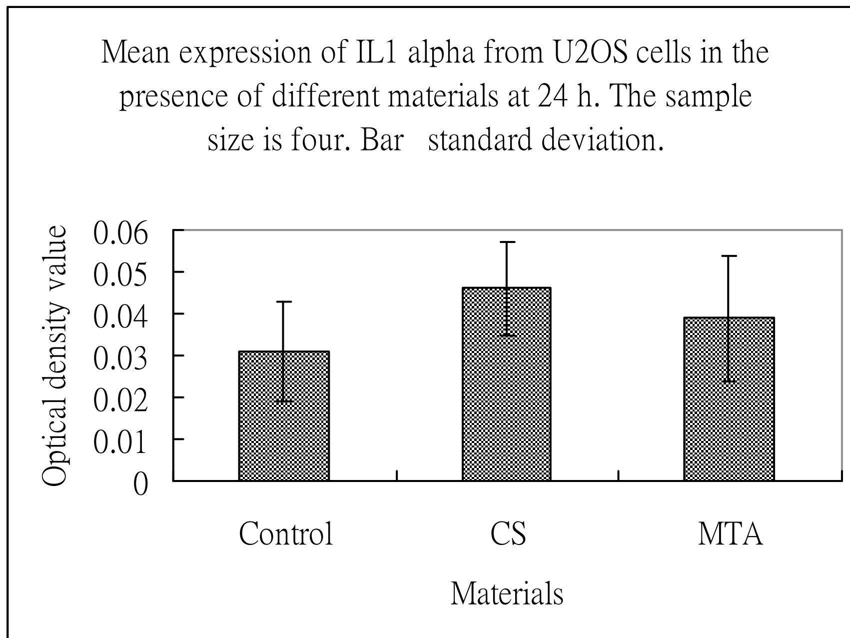


Figure 4. Mean expression of IL-1 beta from U2OS cells in the presence of different materials at 24 h. The sample size is four. Bar ± standard deviation.

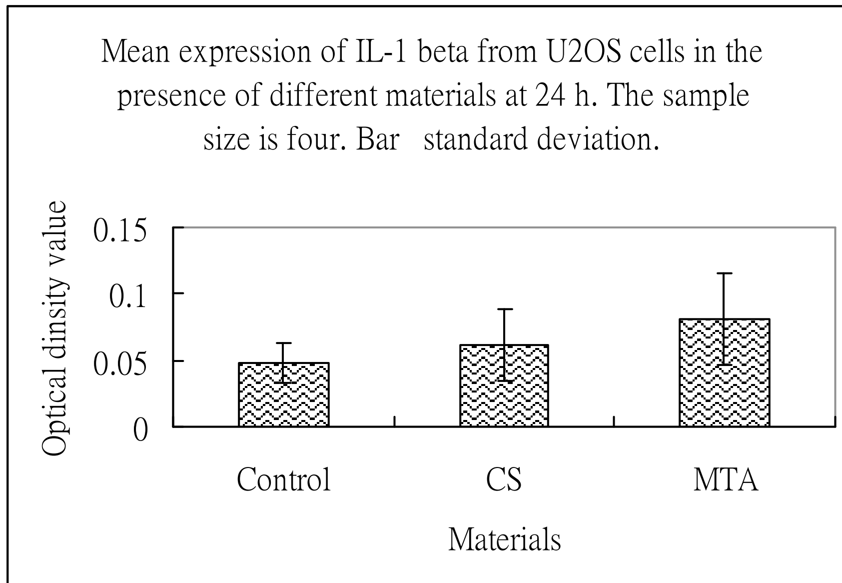


Figure 5. Mean expression of IL-6 alpha from U2OS cells in the presence of different materials at 24 h. The sample size is four. Bar ± standard deviation.

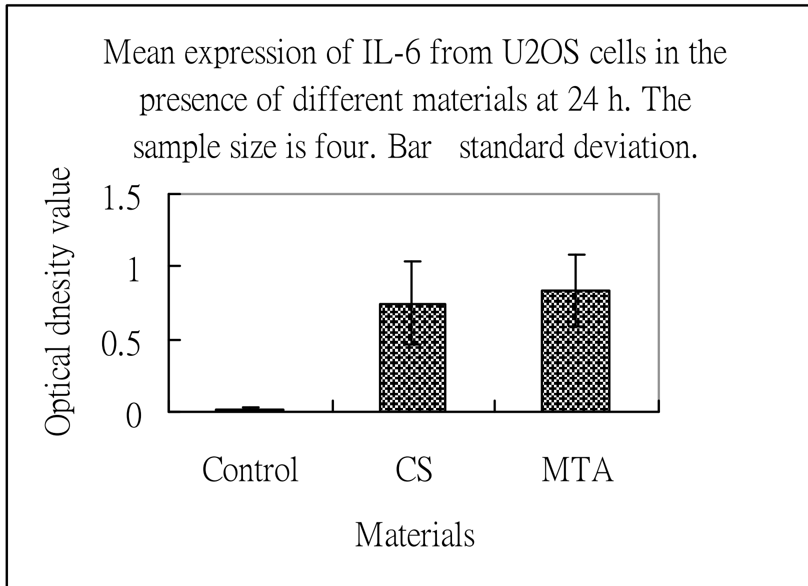


Figure 6. (A) The XRD pattern of hydrated MTA and CS cement mixed with distilled water. The C3S (3CaO.SiO2), C2S (2CaO.SiO2), and C3A (3CaO.Al2O3) peaks were identified at 2q=27.3_ and 2q=32_ to 34_. The two groups have similar major constituents. (B) The survival rate of an MTA and CS cement treated MG63 cell line. Both are shown to be biocompatible with MG63 cells. (C) The cellular proliferation signal ERk and p-ERK kinase expression of MTA and CS cement treated MG63 cells. High ERk and p-ERK kinase expression were noted.

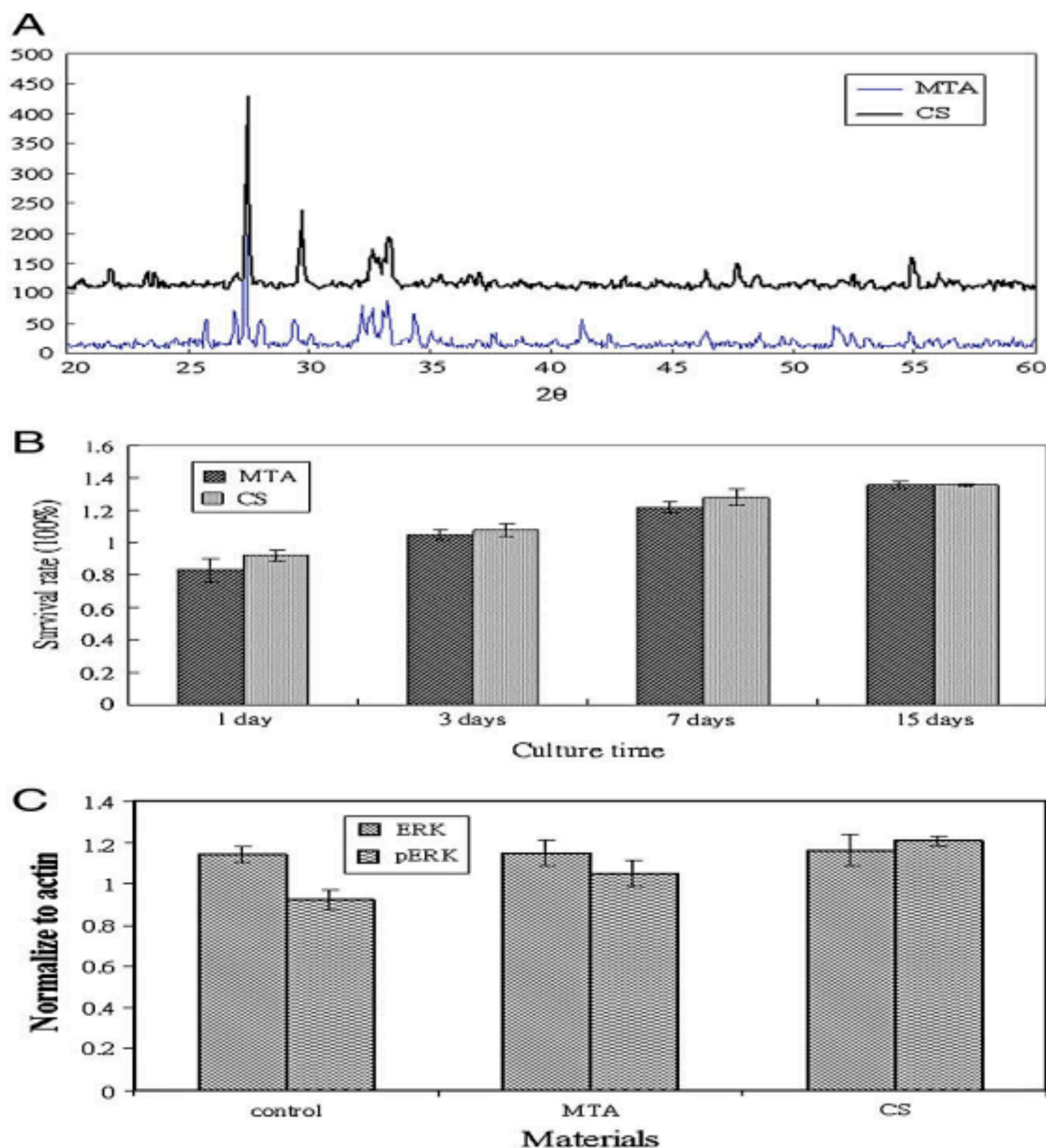


Figure 7. Bone marker expression in the MG63 cell line treated with MTA and CS cements. MTA1, mineral trioxide aggregate after 1 day; MTA3: after 3 days; MTA7, after 7 days; MTA15, after 15 days. CS1, calcium silicate cement after 1 day; CS3, after 3 days; CS7, 7 days; CS15, 15 days; Col, type I collagenase; OC, osteocalcin; ALP, alkaline phosphatase; BSP, bone sialoprotein; OPN, osteopontin.

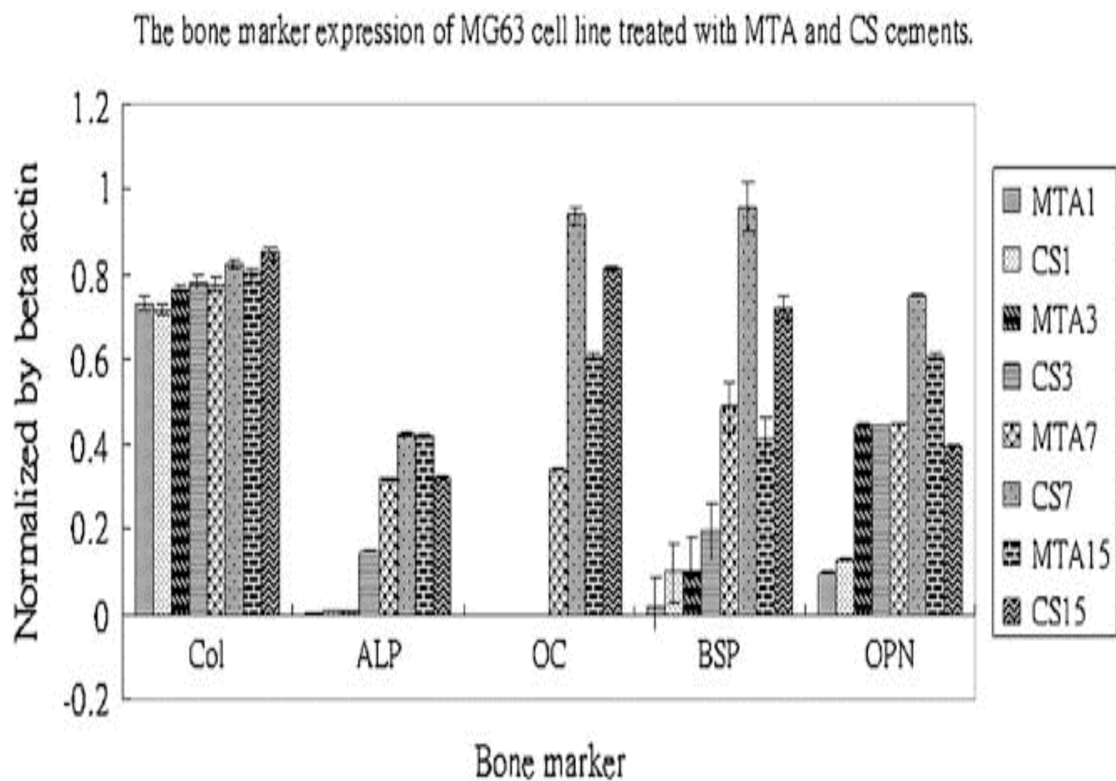


Figure 8. The inflammation reaction of the CS implant

	1wk	6 wk	12wk
Subcutaneous	1.63 ± 0.31	2.26 ± 0.41	1.72 ± 0.53
Parietal bone	1.21 ± 0.42	1.89 ± 0.54	1.76 ± 0.46

Figure 9. The inflammation reaction of the MTA implant

	1wk	6 wk	12wk
Subcutaneous	1.78 ± 0.44	2.88 ± 0.54	1.61 ± 0.51
Parietal bone	1.10 ± 0.37	1.63 ± 0.49	1.87 ± 0.42

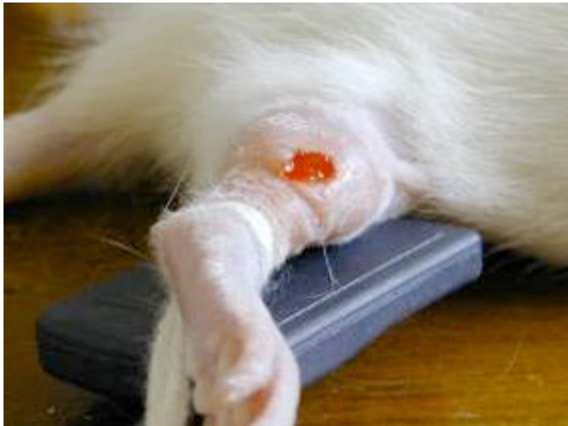
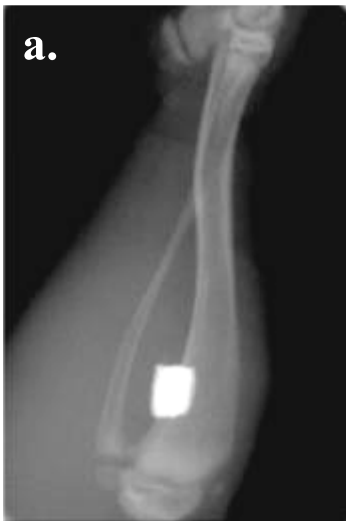
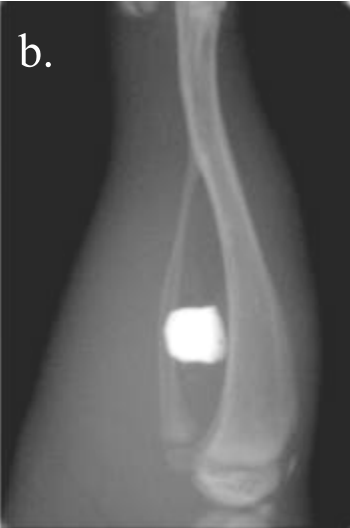
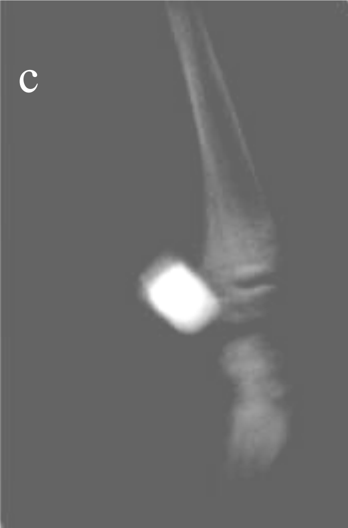


Fig 10. Radiology examination of root-end filling-material MTA implantation a). 1 wk b) 6wks c) 12 wks d) control group.







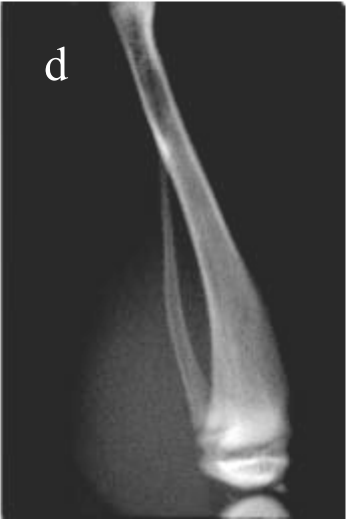
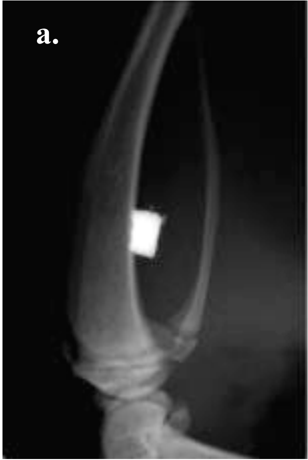
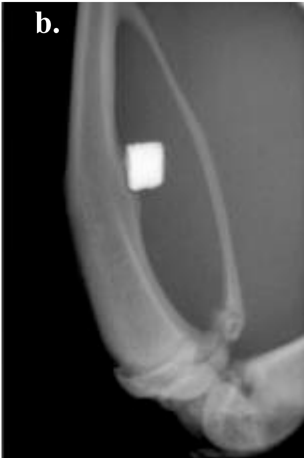
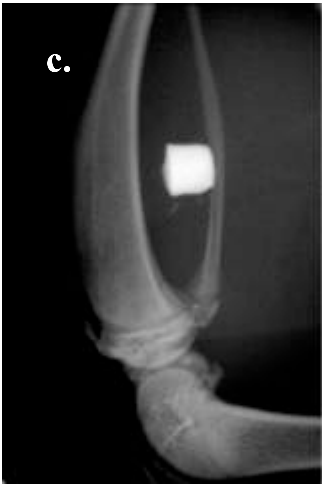


Fig 11. Radiology examination of root-end filling-material CS implantation a). 1 wk b) 6wks c) 12 wks d) control group.







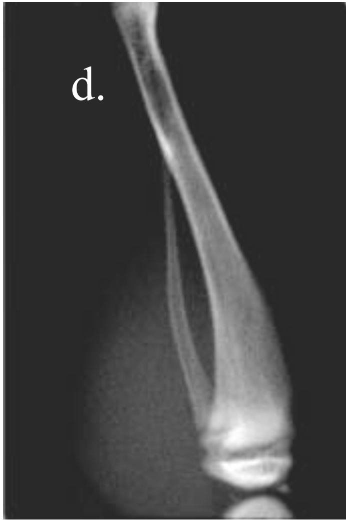


Fig I2. Histological observation of root-end fillers (MTA) implanted on the muscle layer of the rat leg (phase contrast microscope, original magnifications x40 and x100, hematoxylin and eosin stain): control group (a);1 wk (b), 6 wks (d), 8 wks (f) at 40 x magnify. 1 wk (c), 6 wks (e), 8 wks (g) at 40 x magnify

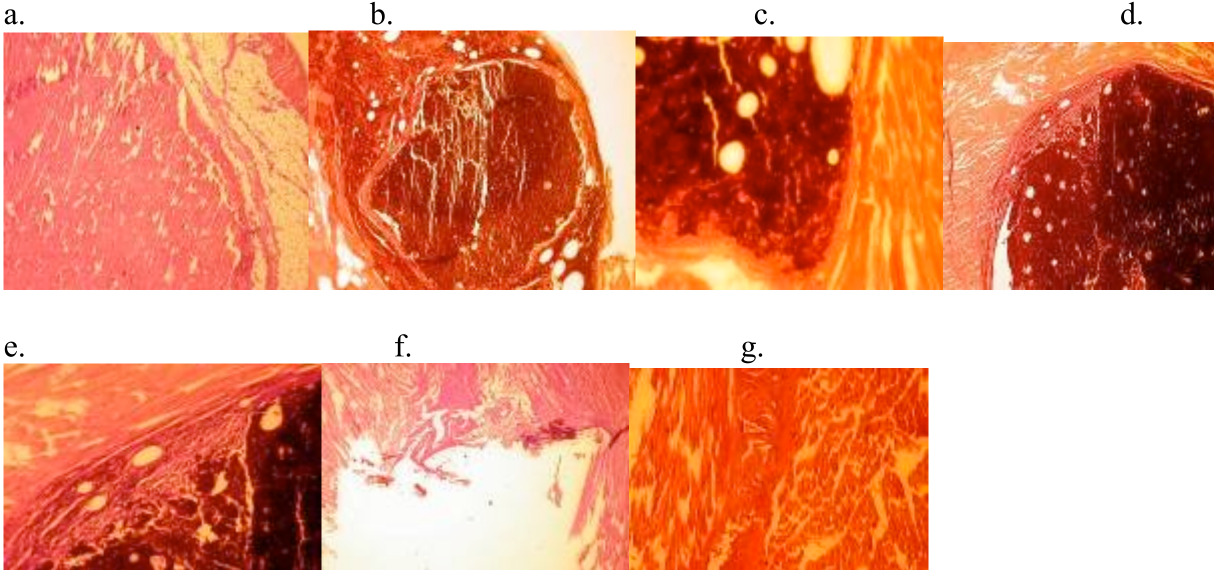
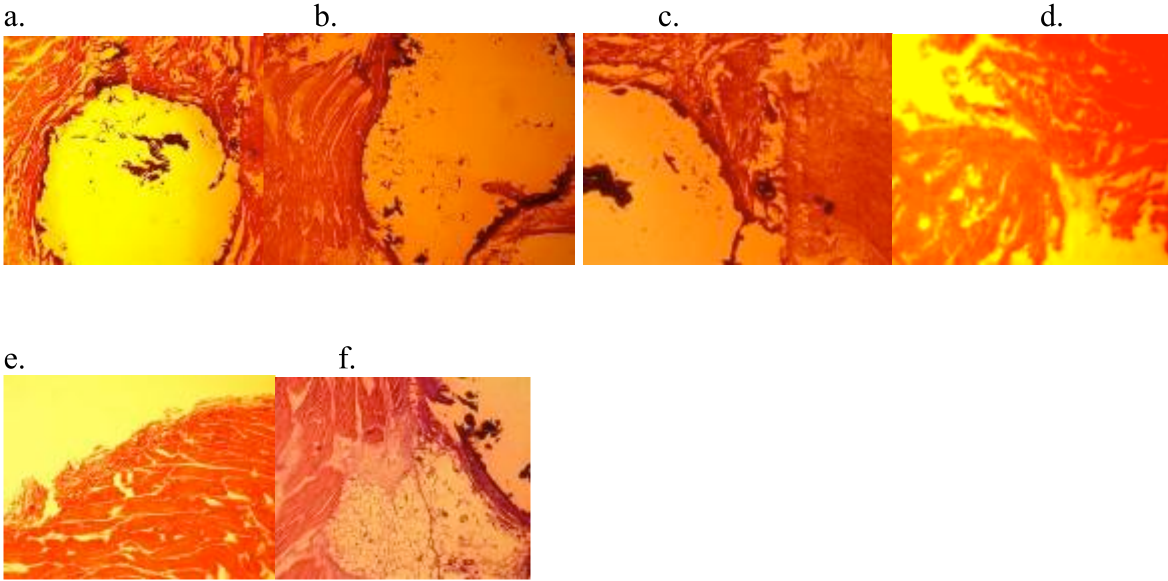


Fig I3. Histological observation of root-end fillers implanted (CS) on the muscle layer of the rat leg (phase contrast microscope, original magnifications x40 and x100, hematoxylin and eosin stain): 1 wk (a), 6 wks (b), 8 wks (c) at 40 x magnify. 1 wk (d), 6 wks (e), 8 wks (f) at 40 x magnify



Figrue 14 Implant on rat skull.

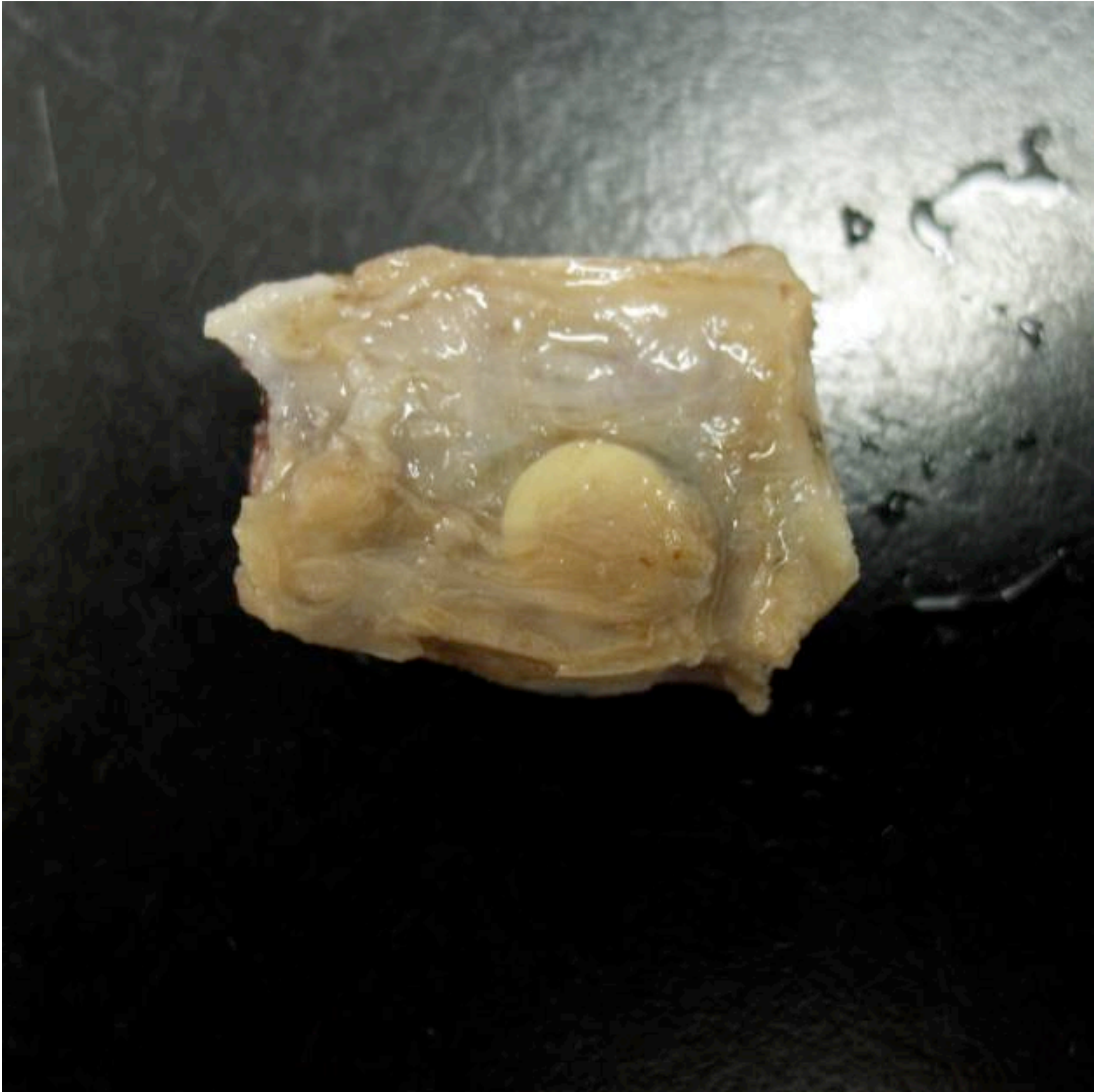


Figure 15 CS Implant on soft tissue. The mateiral is white embaded in tissue.

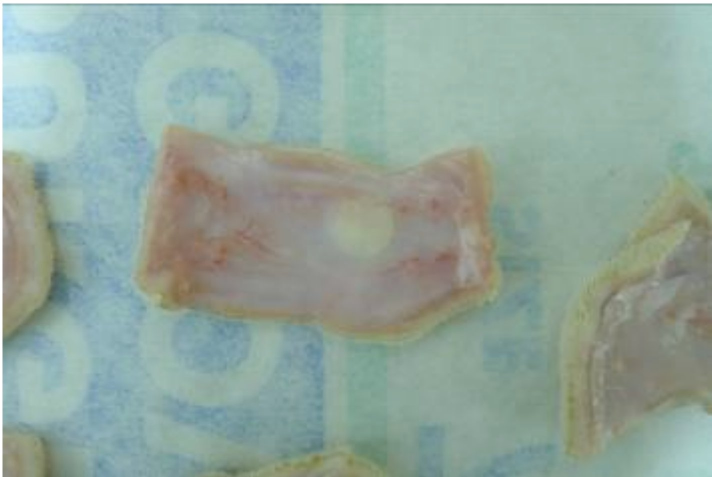
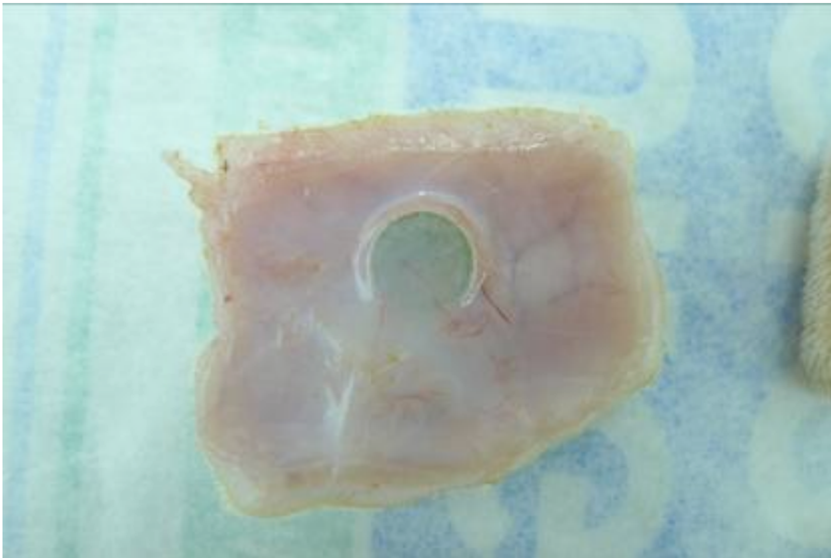


Figure 16. The MTA implant in soft tissue, the mateiral showed gray color.



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）

本研究計畫為針對自己實驗室合成之鈣矽類材料作生物相容性之研究與探討。學術上、我們的材料與商品化ＭＴＡ材料為正控制組比較，發現此類鈣矽材料具有優良之生物相容特性，更發現具有骨頭細胞傳導功能，對於骨頭修復有幫助。其意義上，由於早期ＭＴＡ為獨賣之產品，價格昂貴且宥具一些缺點，我們開發此材料，略作些改質，其性質與效果均表現良好，因此價值應用上應該可以用於臨床上，唯仍須進行人體試驗後，才可以真正幫助臨床上之使用

出席國際會議心得報告書

案號： 97-2314-B-040-021-MY3　　　　**100年03月16-19日**

<table>
<tr><td>報 告 人</td><td>黃翠賢</td><td>單位</td><td>中山醫學大學</td></tr>
<tr><td rowspan="2">會議名稱</td><td>中文：第八十九屆世界牙醫學會(IADR)學術年度大會</td><td rowspan="2">會議地點</td><td>國家：美國</td></tr>
<tr><td>英文：89th International Association of Dental Research Annual Meeting</td><td>城市：San Diego</td></tr>
<tr><td rowspan="2">發表論文題目</td><td colspan="3">中文： 低能二極體雷射降低細菌外毒素誘導骨細胞發炎</td></tr>
<tr><td colspan="3">英文： Low level diode laser therapy reduces lipopolysacharride (LPS) induced bone cell inflammation</td></tr>
</table>

心　　得　　報　　告

一、參加會議經過

今年IADR於過去六年前舉辦過之美國聖地牙哥市舉辦，由於過去此地辦過此會議，加上剛好碰上日本大地震與海嘯，因此似乎參加會議之非美國本人士較過去少。但是，會場中也碰到不少來自日本之友人，也急忙問候有關日本災難後之現況，並給與他們安慰與鼓勵。

會議過程，與本校同事一起第一天出發由台北起飛直飛洛杉磯，再由洛杉磯轉到聖地牙哥，經過近二十小時之路程，抵達時大家都感疲憊。為避免報到時人潮過多，當下即前往會場先作報到確認工作。今年由於我們中部醫師似乎都未接到預先要寄來之名牌，因此於會場上耗費許多時間處理，也顯示今年之舉辦單位準備上較不周全。由於位於全美最佳生活之都市，此會場位於海邊，佔地空曠，景色宜人，休閒氣份濃厚。

第二天為本人之論文發表，一早隨即趕在八點去貼海報，進會場時發覺野蠻多醫師已經都貼上海報，今年參與會議之論文3677篇，較去年少許多，貼完之後參觀了鄰近之海報內容，內容水準上不錯，海報製作也達一定品質。由於下午才須於海報現場，因此參觀了今年度的廠商商展，發現似乎較往年少的攤位擺設，新的產品或研究設備未見擺設。下午須站於海報前，本人今年報告內容也吸引同好醫師之討論，提供本人許多寶貴經驗。

第三天去會場， 因有學生的海報發表，同樣一早多即去貼海報論文，見到各國參加IADR Hatton competition的海報，有的非常偏基礎，有的也淺顯易懂，各領域皆有，品質方面表現很好。之後，中午時，去聽牙材的口頭演講，內容有關材料毒性。 今日與會人數較多，不過發現有些醫師當口頭報告後，隨即一群人消失離去，發現很多人是為同事來捧場的。

第四天 同樣一早八點多即去會場，因有學生的海報發表，同樣一早多即去貼海報論文，空餘時間至中午海報展示開始前，順便選擇聆聽一些相關於自己研究之

出席國際會議心得報告書

案號：97-2314-B-040-021-MY3　　　　**100年03月16-19日**

口頭報告。

第五天 因是最後一天下午，參加的人數明顯變少，但有些主題還是值得聆聽。因此無參加聆聽口頭報告外，順到再與一些國際友人聊些研究方向。下午之後，許多人都已陸續離開，結束今年度之盛會。

二、與會心得

每年三、四月或是六七月都有IADR會議之舉辦，今年度在聖地牙哥舉辦，由於是渡假區，許多醫師來此順道作一休閒旅遊，雖然人潮不如去年熱烈，但是熱門主題之聽眾還是很多人，沒有提早去還不一定有位置座，如華人再幹細胞之研就上有名之醫師黃子舟、施松濤等，其報告上就有許多醫師發問，似乎也顯示口腔醫學之研究發展上，幹細胞之應用已經為一趨勢。

每年都會有來自世界各地之口腔醫學研究人才來此發表，雖然今年剛好遇上日本地震災難，同好間遇上日本友人，還是給與許多的祈禱與祝福，也給與精神上或是物質上之支持。對於日本發生如此重大災難，他們仍勇敢出來參加此盛會，其學術之精神上更是值得敬佩。

個人每年都會帶領自己的研究生出席，讓這些學生作一學習，也就地取材順便作一些教學，灌輸學生有機會要出來參與論文競賽，此讓研究生也見識到世界上做研究的風氣，台灣研究生應該如何應付，才不至於讓台灣的學術研究品質落後。一樣的，每次都可以見到會議上總有遇見來自台灣之不同單位醫師，有的是人數稍多的團體，有的是三四個一起參加，晚上時段，國外一些學校團體常有某某晚會等活動，今年仍就不見台灣有此類似活動，就連開幕式會上只有台大林院長與陽明許主任代表，感覺稀落。還是一同往常希望國內牙醫團體組織可以贊助一些活動，邀請一些外賓參加，順道進行國際交流。

三、建議

1. 建議有一設常單位負責帶領國內研究人員參與此會。
2. 鼓勵政府提供更多研究生之補助，鼓勵多多參加。

四、攜回資料名稱及內容（附件：與會手冊封面、論文暨海報發表時程等影本）

帶回大會手冊封面、論文暨海報發表時程等，今年採USB裝置下載摘要，無紙本。

Abstract

569 Low-level diode laser therapy reduces lipopolysaccharide (LPS)-induced bone cell inflammation

Thursday, March 17, 2011: 2 p.m. - 3:15 p.m.

Location: Hall C (San Diego Convention Center)

Presentation Type: Poster Session

T.-H. HUANG, *C.-T. KAO, and Y.-C. LU, Chung Shan Medical University, Taichung, Taiwan*

Objectives: The aim of this study was to investigate the cytologic effects of inflammatory bone cells after in vitro low-level laser therapy (LLLT). Methods: A human osteosarcoma cell line (MG63) was cultured, infected with lipopolysaccharide (LPS) and exposed to low-level laser treatment at 5 J/cm2 or 10 J/cm2 using a 920 nm diode laser. MG63 cell attachment was observed under a microscope, and cell viability was quantified by mitochondrial colorimetric assay (MTT). LPS-treated MG63 cells were irradiated with LLLT, and the inflammatory markers, iNOS, TNF-α and IL-1, were analyzed by reverse transcription polymerase chain reaction (RT-PCR) and western blot. The data were collected and analyzed by one-way analysis of variance (ANOVA); $p<0.05$ indicated a statistically significant difference. Results: Low-level laser treatment on MG63 cells increased their ability to attach and survive. After irradiation, the expression levels of iNOS, TNF-α and IL-1 in LPS-infected MG63 cells decreased over time ($p<0.05$). Conclusions: Low-level diode laser treatment increased the proliferative ability and decreased the expression of inflammatory mediators in MG63 cells.

Introduction

Low-level laser therapy (LLLT) has applied more in regenerative medicine and dentistry [1]. Photodynamic therapy is become a feasible medical technology in the 1980s. Laser diode is formed by doping a very thin layer on the surface of a crystal wafer. It is also known as an injection laser. Laser diodes differ from conventional lasers, such as small size and weight, low current, low intensity and wide-angle beam. It is reported that LLLT appears to increase mitochondrial respiration and adenosine triphosphate (ATP) synthesis [2, 3] and possesses biostimulatory effects on wound healing, collagen synthesis [4], and fibroblast proliferation [5,6]. Literatures review showed that LLLT can accelerate bone formation by increasing osteoblastic activity, vascularization, organization of collagen fibers and ATP levels [7-9].

LLLT demonstrated with anti-inflammatory, analgetic and regenerative effects [10,11]. The photodynamic therapy can reduce the numbers of viable bacteria in periodontal diseases [1]. In orthodontic tooth movement, LLLT can reduce patient pain without interfering with the tooth movement [12]. The orthodontic pain is

derived from the secretions of inflammatory cell that stimulate the nerve. The release of pro-inflammatory cytokines and lysosomal enzymes can promote tissue resorption at orthodontic tooth movement compression site [13]. Prostaglandins, IL-1, IL-6, TNFα, and receptor activator of nuclear factor kappa B ligand (RANKL) are all elevated in the periodontal ligament during tooth movement [13]. Thus it can applied LLLT to reduce the orthodontic pain and increase the tooth movement.

The purpose of present study was to investigate the cytologic effects of inflammatory bone cell after LLLT treatment in vitro.

Materials and Methods

Laser conditions

The diode laser (Ga-Al-As , Arts-Laser. Arts International Biotechnology Inc. German) 920 nm was used in this study. The beam was clipped to cover the entire area of the plate (3.3 cm^2). On average, a power output of 0.5W was measured, and this was calculated to deliver 5 J/cm^2 and 10 J/cm^2 (experimental group). Non-irradiated (0 $J/cm^{2)}$ cells were used as control group and were kept under the same conditions. Both irradiated and non-irradiated samples were re-incubated at 37°C in a humidified atmosphere of 5% CO_2.

Immunofluorescent stain

MG63 cells were seeded to adhere to the 3 cm dish (Techno Plastic Products, 92006, Germany) at a density of 5 $x10^4$ cell/mL for 24 hours. The lipopolysacharride (LPS, Sigma Co.) were added to culture for four hours. Cultured MG63 were irradiated with the lids off in the dark, at room temperature, with 5 J/cm^2 and 10 J/cm^2 at 920 nm. Non-irradiated cells were used as controls and were kept under the same condition. After tone hour and 12 hours cells were fixed with 10% methanol (Sigma, St Louis, MO) for 20 minutes. Samples were blocked in PBS supplemented with 5% bovine serum albumin (Sigma) and then incubated in blocking buffer containing rabbit anti-phospho-FAK primary antibody (FAK; Abcam, Interlab Co, Ltd, Taipei, Taiwan) (1:1000 dilution in PBS) and iNOS antibody (rabbit; Invitrogen). After washing the cells with PBS, the cytoskeleton and cell nucleus were stained by incubating the cells with the secondary antibody (Biotin-conjugated goat anti-rabbit antibody) and phalloidin- FITC (Invitrogen) (1:1000 dilution in PBS), for 1 hour and with DAPI (Invitrogen) (30 nmol/L in PBS) for 20 minutes, respectively [15]. The stained cells were visualized by using a Zeiss (Carl Zeiss, Oberkochen, Germany) fluorescent

microscope.

Western blot assay

The Western blot analysis is used to determine the expression of MG 63 cell iNOS and pErk markers at 1 h, 3 hr and 12 hr after irradiation. According to our previous method [16], cultured cells were lysed in lysis buffer [RIPA lysis buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 150 mM NaCl，1 mM EDTA，1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitor cocktail tablet)] on ice. Cell extracts were sonicated, and protein concentration was determined with a Coomassie® Brillrant blue G-250 (Bio-Rad, 500-0006, US). Ten micrograms of protein were loaded in each lane. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred to polyvinylidene diflouride membranes, (Immunoblot PVDF membrane, BioRad, 162-0177).

Membranes were blocked overnight in blocking buffer containing Tris-buffered saline [TTBS—50 mM Tris; 150 mM sodium chloride (NaCl), Separation Scientific, 7647-14-5] containing 0.1% Tween 20 and 5% non-fat milk. The membranes were then incubated in primary antibody [rabbit IgG anti-human iNOS (1:1000,upstate,07-520,US)、rabbit IgG anti-phospho-ERK (1:1000,cell signaling)、mouse IgG anti- β-actin (0.5:1000, Chimecon,MAB1501,US)] diluted in blocking buffer (as above) at room temperature for 1 h. The membranes were washed in TTBS and then incubated in secondary antibody [HRP-conjugated goat anti-rabbit IgG (1:5000, Chemicon, AP132P)、HRP- conjugated goat anti-mouse IgG (0.5:5000, Chemicon, AP124P)] diluted in blocking buffer (as above) at room temperature for 1 h. The membranes were washed and stained with 1X TMB/H (3,3’,5,5’-tetramethylbenzidine, Chemicon, ES014). The blots were then exposed to Xray film (Kodak MXG, Rochester, USA, 326052). The films were developed and then used BioDoc system to take record and LabWorks 4.5 soft ware to detect the bend density.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay

MG63 cells were seeded to adhere to the 3 cm dish (Techno Plastic Products, 92006, Germany) at a density of 5 x10^4 cell/mL for 24 hours. The lipopolysacharride (LPS, Sigma Co.) were added to culture for four hours. Cultured MG63 were irradiated with the lids off in the dark, at room temperature, with 5 J/cm^2 and 10 J/cm^2 at 920 nm. Non-irradiated cells were used as controls and were kept under the same condition.

Procedures were followed our previous experiment method [16]. Total RNA was isolated according to the manufacturer's instructions. MG63 cell were harvested by 0.25% tripsin-EDTA and extracted with TRIZOL reagent (Invitrogen, 15596-018, India) 10 minutes. Added 200 μl chloroform (Tedia, CR-0360, US) 10 minutes, at 12,000 rpm, 4 ^{o}C centrifugation for 15 minutes. Added 500 μl isopropanol (Fluka, USA) to supernatant 10 minutes, at 12,000 rpm, 4 ^{o}C centrifugation for 15 minutes. Remove the supernatant and washed with -20 ^{o}C 75% alcohol twice. The RNA in aqueous solution was reserved in -20 ^{o}C and detected by spectrophotometry ate 260nm.

1 μg of total RNA in aqueous solution was reverse transcribed in a volume of 50 μl containing 400 μM of each NTP, 10 units of RNase inhibitor, 10 μl 5 x Tris buffer, 2 μl RT-PCR enzyme mix and 800 nM of TNF α and IL 1 primer.

Reverse transcription was carried out at 50 °C for 30 min. After an initial denaturation step at 95 °C for 15 min, 40 PCR cycles were run, each consisting of denaturation at 94 °C for 1 min, annealing for 1 min at the respective temperature, extension at 72 °C for 1 min, and finally elongation at 72 °C for 10 min. The PCR products were separated by electrophoresis of 20 μl of each reaction mixture in a 2% agarose gel at 100 V/cm in 1 % Tris acetate EDTA buffer. Following electrophoresis, the gels were stained with thidium bromide, destained in distilled water and photographed with a charge-coupled device camera. The Lab Works 4.5 soft ware was used to analysis band density.

All above measurements were made in duplicate and are expressed as mean ± SD of three independent experiments. In addition, one-way ANOVA and Tukey's multiple comparison test were used to reveal significant differences between the different investigation groups. The level of significance was set at $P < 0.05$.

Results

MG63 cell attachment and viability of attached cells

At each time interval, cell density and distribution were examined by light microscopy. It is evident that the first or second hour observation of MG63 attachment was no obviously difference. The MG63 cell attachment were higher in 5 J/cm^2 and 10 J/cm^2 irradiated groups at 12 hours (Fig. 1).

In all samples the number of attached viable cells increased during the cultivation period. After one hour and twelve hour observation, the cell viability existed statistical difference ($p<0.05$) between the control and experimental group (Fig.2).

iNOS expression of MG63

In fluorescent stain of MG63 treated with different LLLT showed iNOS expression decreased (Fig. 3). After one hour of LLLT showed red dots (iNOS marker) were decreased as laser power was increased (Fig. 3). After 12 hour of LLLT showed no iNOS expression in irradiated groups (Fig.3).
The irradiated groups' iNOS expression were lower than control group after one hour and three hour time period ($p<0.05$) but no difference after 12 hours ($p>0.05$) in western bolt assay (Fig.4).

pErk expression of MG63
The MG 63 cell's pErk expression showed irradiated groups were higher than non-irradiated group at different time period ($p<0.05$, Fig. 5).

TNF-α and IL 1 markers expression of MG63
The inflammatory markers TNF-α and IL-1expression were shown in figure 7 and 8. The TNF-α expression level were lower in irradiated groups ($p<0.05$) after 1 or 12 hour treatment (Fig. 6).
The IL-1 expression level was lower in irradiated groups after 12 hour treatment ($p<0.05$), but found no difference between irradiated and non-irradiated groups in one hour treatment ($p>0.05$, Fig. 7).

Discussion

The present in vitro study evaluate the effects of LLLT on inflammatory MG63 cells and showed that LLLT on adherent and viability of MG63 cells were higher in cells treated with 5 J/cm^2 and 10 J/cm^2 . It is also found that LLLT can reduce the inflammatory markers iNOS, TNF- and IL-1 expression in infected MG63 cells.
The viability assay was irradiated after MG63 cell attached to dishes. The results showed that after irradiated 12 hours, the MG63 viability was significant difference with control ($P<0.05$ Fig. 2). It represented that LLLT do have biostimulatory effects. The present study showed similar results as previous studies findings that LLLT can enhancement of fibroblast and chondral proliferation (17,18). This result also demonstrated from present pErk marker expression (Fig.5).
The higher expression of pErk signal showed cell was in high proliferation state (19). LLLT of diode laser can promote the cell to growth in present study.
To understand the effects of LLLT on inflammatory cell, present study applied the LPS into cell culture. This method is seldom seen in present LLLT studies. Lipopolysaccharide (LPS) is one of the most powerful bacterial virulence factors in terms of proinflammatory properties (20). Endotoxin, a cell wall component of

Gram-negative bacteria, plays a central role in the pathogenesis of septic shock. In conjunction with these changes in organ function, a wide variety of inflammatory mediators are released which appear to contribute to these responses. These include the release of proinflammatory cytokines (e.g. tumor necrosis factor-alpha, IL-1 beta, IL-6, IL-8) (21). In present study, to prove LLLT has anti-inflammatory activity (22), author compared TNF-α and IL-1expression of the LLLT effects on LPS induced inflammation MG63 cells. The present result agree with it and showed diode laser with 5 J/cm^2 and 10 J/cm^2 irradiation to inflammation MG63 can reduce the TNF-α and IL-1expression as compared with control (Fig. 6 and 7).

In inflammation, proinflammatory cytokines and lipid mediators play an important role in triggering the expression of the inducible isoform of nitride oxide (NO) synthase (iNOS or NOS-2) in various types. It is proved that proinflammatory cytokines, such as the TNF-α and IL-1 can induce the expression of INOS in vitro or in vivo (23). Platelet activating factor (PAF) can induce iNOS and enhance lipoploysaccaharide (LPS) induced expression of iNOS in macrophages (24). With this mechanism, the present study was using LPS to induce the cell inflammation and detect iNOS expression by fluorescent analysis and western blot analysis. The results showed MG 63 cell after LLLT treated, the iNOS fluorescent strength were decreased (Fig.3) and iNOS protein expression were decreased too (Fig. 4). It is support that LLLT has anti-inflammation effects.

In dentistry, tissue engineering of bone is an important field in periodontal disease, implantology orthodontic tooth movement. This concept is an exciting technology in regenerative medicine. LLLT phototherapy can provide the biostimulation effects to tissue. To enhance using the LLLT in clinical application will be beneficial to patient.

Conclusion

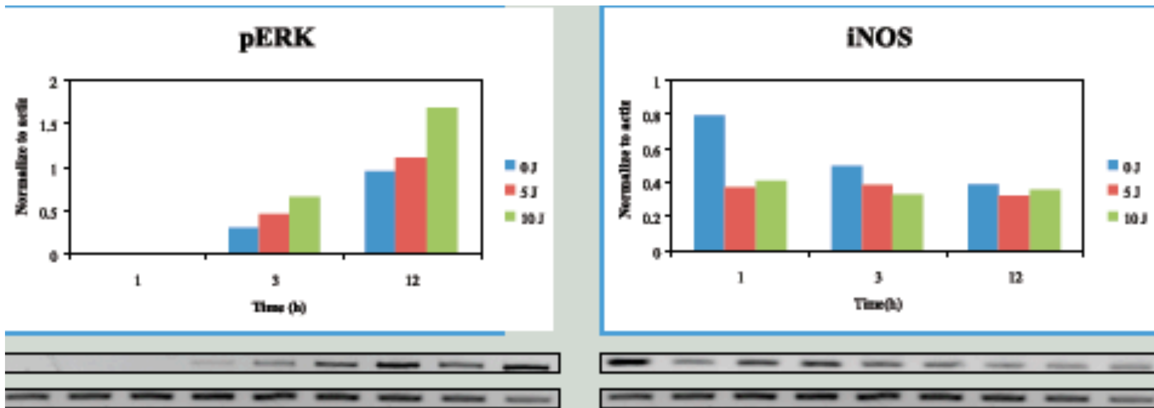
By applying the diode laser with low level power can promote the cell adhesion, increase the MG63 cell proliferation, reduce LPS induce inflammation markers iNOS, TNF-α and IL-1 expression.

Reference

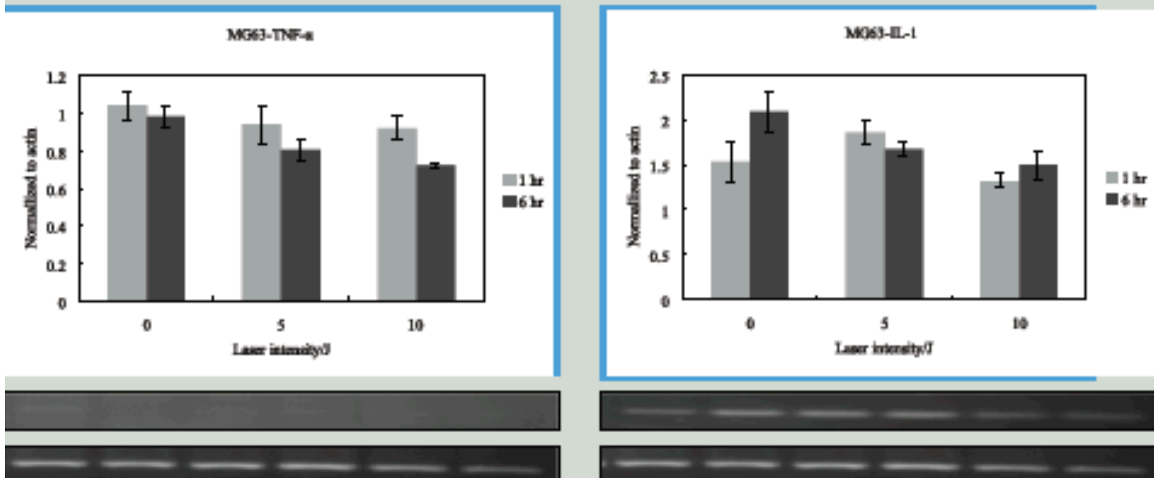
1. Pinheiro SL, Donegá LM, Seabra LMS, Adabo MD, Lopes T, Dias do Carmo T H, Ribeiro MC, Bertolini PFR (2010) Capacity of photodynamic therapy for microbial reduction in periodontal pockets Lasers Med Sci 25:87–91.

2. Morimoto Y, Arai T, Kikuchi M, Nakajima S, Nakamura H (1994) Effect of low-intensity argon laser irradiation on mitochondrial respiration. Lasers Surg Med 15:191–199.
3. Ninomiya T, Hosoya A, Nakamura H, Sano K, Nishisaka T, Ozawa H (2007) Increase of bone volume by a nanosecond pulsed laser irradiation is caused by a decreased osteoclast number and an activated osteoblasts. Bone 40:140–148.
4. Conlan MJ, Rapley JW, Cobb CM (1996) Biostimulation of wound healing by low-energy laser irradiation. J Clin Periodontol 23:492–496.
5. Pourzarandian A, Watanabe H, Ruwanpura SM, Aoki A, Ishikawa I (2005) Effect of low-level Er:YAG laser irradiation on cultured human gingival fibroblasts. J Periodontol 76:187–193.
6. Almeida-Lopes L, Rigau J, Zangaro RA, Guidugli-Neto J, Jaeger MM (2001) Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. Lasers Surg Med 29: 179–184
7. Lirani-Galvão AP Jorgetti V, Lopes da Silva O (2006) Comparative study of how low-level laser therapy and low-intensity pulsed ultrasound affect bone repair in rats. Photomed Laser Surg 24:735–740
8. Karu T, Pyatibrat L, Kalendo G (1995) Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro. J Photochem Photobiol B 27:219–223
9. Usuba M, Akai M, Shirasaki Y (1998) Effect of low level laser therapy (LLLT) on viscoelasticity of the contracted knee joint: comparison with whirlpool treatment in rats. Lasers Surg Med 22:81–85.
10. Turhani D, Scheriau M, Kapral D, Benesch T, Jonke E, Bantleon HP (2006) Pain relief by single low-level laser irradiation in orthodontic patients undergoing fixed appliance therapy. Am J Orthod Dentofacial Orthop 30: 371–377.
11. Kon K (1992) In vivo study of low power laser irradiation on new bone formation of rat calvaria defect. J Jpn Soc Laser Dent 3: 35–44
12. Fujiyama K, Deguchi T, Murakami T, Fujii A, Kushima K, Tanakno-Yamamoto T. (2008) Clinical effect of CO2 laser in reducing pain in orthodontics. Angle Orthodontist 78:299-303
13. Yamaguchi M, Kasai K. Inflammation in periodontal tissues in response to mechanical forces. ArchImmunol Ther Exp (Warsz) 2005;53:388–398.
14. Huang TH, Ding SJ, Kao CT (2007) Biocompatibility of various formula root filling materials for primary teeth. J Biomed Mater Res Part B Appl Biomater 80B:486–90.

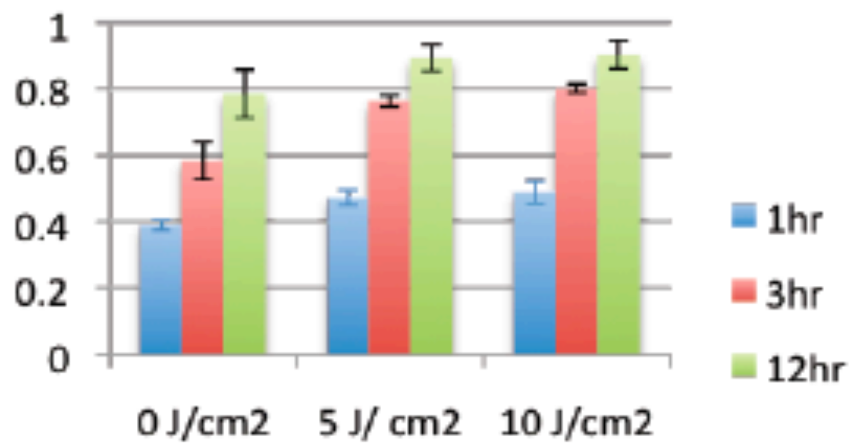
15. Chen CL, Kao CT, Ding SJ, Shie MY, Huang TH (2010) Expression of the inflammatory marker Cox-2 in dental pulp cells cultured with mineral trioxide aggregate (MTA) or calcium silicate (CS) cements. J Endod 36:465-468.
16. Chen CL, Huang TH , Shie MY, Ding SJ, Kao CT (2009) Comparison of Calcium and Silicate Cement (CS) and Mineral Trioxide Aggregate (MTA) Biologic Effects and Bone Markers Expression in MG63 Cells. J Endod 35:681-685
17. Boulton M, Marshall J (1986) He-Ne laser stimulation of human fibroblast proliferation and attachment in vitro. Lasers Life Sci 1: 123–134.
18. Schultz RJ, Krishnamurthy S, Thelmo W, Rodriguez JE, Harvey G (1985) Effects of varying intensities of laser energy on articular cartilage. Lasers Surg Med 5: 557–588.
19. Huang TH, Ding SJ, Hsu TC, **Kao CT (2003)** Effects of mineral trioxide aggregate (MTA) extracts on mitogen-activated protein kinase activity in human osteosarcoma cell line (U2OS). **Biomaterial** 24: 3909-3913
20. Blais DR, Vascotto SG, Griffith M, Altosaar I. (2005) LBP and CD14 secreted in tears by the lacrimal glands modulate the LPS response of corneal epithelial cells. Invest Ophthalmol Vis Sci. 46(11):4235-44.
21. Martich GD, Boujoukos AJ, Suffredini AF. (1993) Response of man to endotoxin Immunobiology. 187(3-5):403-16.
22. Honmura A, Yanase M, Obata J, Haruki E (1992) Therapeutic effect of Ga-Al-As diode laser irradiation on experimentally induced inflammation in rats. Lasers Surg Med 12: 441–449.
23. Thiemermann C. (1997) Nitric oxide and septic shock. General Pharmacology: The Vascular System .29: 159-166.
24. Howard A.D.; Erickson K.L . (1996) Alteration of Macrophage Responsiveness to Platelet-Activating Factor by Interferon-gamma and Lipopolysaccharide. Cellular Immunology 174:155-164.



The results showed that application of LLLT on LPS infected MG63 cells can increase cell proliferation (pERK high expression).



After irradiation, the expression levels of iNOS, TNF-α and IL-1 in LPS-infected MG63 cells decreased over time ($p<0.05$).



The MG63 cells attachment showed statistic difference between control and experimental group ($p<0.05$).

國科會補助計畫衍生研發成果推廣資料表

日期:2011/09/11

<table>
<tr><td rowspan="3">國科會補助計畫</td><td>計畫名稱: 矽鈣合成物於體外與體內之生物活性研究</td></tr>
<tr><td>計畫主持人: 黃翠賢</td></tr>
<tr><td>計畫編號: 97-2314-B-040-021-MY3　　　學門領域: 牙醫學</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
</table>

97 年度專題研究計畫研究成果彙整表

<table>
<tr><td colspan="3">計畫主持人：黃翠賢</td><td colspan="5">計畫編號：97-2314-B-040-021-MY3</td></tr>
<tr><td colspan="8">計畫名稱：矽鈣合成物於體外與體內之生物活性研究</td></tr>
<tr><td colspan="3" rowspan="2">成果項目</td><td colspan="3">量化</td><td rowspan="2">單位</td><td rowspan="2">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）</td></tr>
<tr><td>實際已達成數（被接受或已發表）</td><td>預期總達成數(含實際已達成數)</td><td>本計畫實際貢獻百分比</td></tr>
<tr><td rowspan="12">國內</td><td rowspan="4">論文著作</td><td>期刊論文</td><td>1</td><td>0</td><td>100%</td><td rowspan="3">篇</td><td></td></tr>
<tr><td>研究報告/技術報告</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>研討會論文</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>專書</td><td>0</td><td>0</td><td>100%</td><td></td><td></td></tr>
<tr><td rowspan="2">專利</td><td>申請中件數</td><td>0</td><td>0</td><td>100%</td><td rowspan="2">件</td><td></td></tr>
<tr><td>已獲得件數</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td rowspan="2">技術移轉</td><td>件數</td><td>0</td><td>0</td><td>100%</td><td>件</td><td></td></tr>
<tr><td>權利金</td><td>0</td><td>0</td><td>100%</td><td>千元</td><td></td></tr>
<tr><td rowspan="4">參與計畫人力（本國籍）</td><td>碩士生</td><td>0</td><td>0</td><td>100%</td><td rowspan="4">人次</td><td></td></tr>
<tr><td>博士生</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>博士後研究員</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>專任助理</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td rowspan="12">國外</td><td rowspan="4">論文著作</td><td>期刊論文</td><td>7</td><td>0</td><td>100%</td><td rowspan="3">篇</td><td>SCI 雜誌 (Journal of Endodntics)</td></tr>
<tr><td>研究報告/技術報告</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>研討會論文</td><td>3</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>專書</td><td>0</td><td>0</td><td>100%</td><td>章/本</td><td></td></tr>
<tr><td rowspan="2">專利</td><td>申請中件數</td><td>0</td><td>0</td><td>100%</td><td rowspan="2">件</td><td></td></tr>
<tr><td>已獲得件數</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td rowspan="2">技術移轉</td><td>件數</td><td>0</td><td>0</td><td>100%</td><td>件</td><td></td></tr>
<tr><td>權利金</td><td>0</td><td>0</td><td>100%</td><td>千元</td><td></td></tr>
<tr><td rowspan="4">參與計畫人力（外國籍）</td><td>碩士生</td><td>0</td><td>0</td><td>100%</td><td rowspan="4">人次</td><td></td></tr>
<tr><td>博士生</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>博士後研究員</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>專任助理</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
</table>

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）

本研究計畫之研究成果陸續投稿發表於 Journal of Endodnotics 和 Journal of Dental Science 等雜誌。

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）

本研究計劃為自行開發合成鈣矽類根間充填材料，於合成後本計劃究材料之物理性質、化學特性、生物相容性與動物植入相容性上，做了試驗。研究成果顯示，雖非技術創新，自行開發合成之材料，其物理性質、化學特性與商品化材料相仿，生物相容性佳，動物試驗結果無排斥或嚴重發炎反應。對於學術上發現此材料有骨誘導效應，商業上，由於製作成本低，比現有商品便宜許多，可於臨床上推廣。