行政院國家科學委員會專題研究計畫 成果報告

研究牙科樹脂牙冠中雙酚A的釋出量
研究成果報告(精簡版)

計 畫 類 別 ： 個別型
計 畫 編 號 ： NSC 99-2314-B-040-015-
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

公 開 資 訊 ： 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10月31日

中文摘要： 雙酚 A(Bisphenol A，也簡稱 BPA)是一種合成塑膠的材料，廣泛的使用在聚碳酸樹脂(polycarbonate)及環氧樹脂(epoxy resins)中，這種塑膠最主要使用在常被使用在食品和飲料的盛具，以及牙科材料上。當我們不經意的使用這些材質的塑膠材料時，雙酚 A 就很可能滲出進入到人體，Bisphenol A 的作用機轉是它會與雌激素受體結合而模仿造成雌激素作用，擾亂了正常人體的作用，發生與雌激素不正常相關的疾病。近期很多有關雙酚 A 的研究，也都認為雙酚 A 是一種很常見並應該避免攝入到人體的內分泌干擾物質。本研究對於台灣牙科診所使用假牙之牙材進行 BPA 溶出濃度之測定。

本研究以四種不同的假牙材料作為實驗分析，分別為 A(Alike)、REB(Rebase)、S2(Structure 2)、SOF(Soft linner)四種不同的材料，每種材料的分析數量為 18 個，共計分析 72 個樣本。將四種不同廠牌牙科樹脂冠浸泡在三種不同溶劑中(水、酒精、醋酸）中，在兩種不同溫度 37 與 65˚C 分別以 24、48、72 小時，收集浸泡液。分別以高效液相層析儀(High performance liquid chromatography 簡稱 HPLC)測量雙酚 A 釋出量。

英文摘要： Bisphenol A (BPA) have become a public concern due to their massive use, ubiquitous occurrence, and persistency in the environment. BPA is mainly used as a monomer in the preparation of polycarbonate plastic and epoxy resins which are used in Various dental materials(dental temporary crown), baby bottles, protective coatings on food containers, and so forth. Bioassay studies have identified that BPA elicited estrogenic potency and chronic toxicity. These compounds enter the food chain in several different ways. In humans, intake of these so- called environmental hormones is mainly via contaminated drinking water and foods.

The purpose of this study was to investigate the release of BPA from dental materials and to evaluate the estrogenic activity of BPA. Dental resin materials were immersed in three solvents (water, ethanol, and acetic acid) at 37 or 65°C for 24, 48 and 72 hs. The elution from the material was analyzed by high-performance liquidchromatography(HPLC) analysis.

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）

研究牙科樹脂牙冠中雙酚 A 的釋出量

(Study Bisphenol A from dental resin crown)

計畫類別：■ 個別型計畫　　□ 整合型計畫

計畫編號：NSC －99－ 2314－B－040－015

執行期間： 99 年 8 月 1 日至 100 年 7 月 31 日

計畫主持人：黃富美

共同主持人：張育超

計畫參與人員：

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

中 華 民 國 100 年 10 月 31 日

前言

雙酚 A (bisphenol A, BPA)，又稱為二酚基丙烷[2,2-bis(4-hydroxyphenyl) propane]，它是由苯酚、丙酮在酸性介質中合成的白色粉末，熔點為 157℃，沸點為 220℃。

BPA 是目前製造聚碳酸酯、環氧樹脂、聚芳酯、酚醛樹脂、不飽和聚酯樹脂和阻燃劑等的重要原料，市面上的聚碳酸酯(Polycarbonate, PC)塑膠產品如嬰兒奶瓶及塑膠水瓶、金屬罐頭內壁的環氧樹脂與牙齒的填充密封劑，都含有 BPA 的成分；BPA 也作為其他種類塑膠之添加劑，如聚氯乙烯(polyvinyl chloride; PVC)包括醫療用的管子、玩具及水管和聚乙烯對苯二甲酸酯(polyethylene terephthalate; PET)包括蘇打和礦泉水的寶特瓶，食品容器之表面 coating 物質之一。

2005 年聯合國環境規劃署建議 16 種環境持久性有機污染產物(Persistant Organic Products, POPs)，其中 12 種為疑似環境荷爾蒙物質，雙酚 A (bisphenol A, BPA)則為其中之一；我國環保署亦於 2007 年將 BPA 列入觀察名單。由於 BPA 廣泛使用在日常生活用品中，包括市面上的聚碳酸酯(Polycarbonate, PC)塑膠產品如嬰兒奶瓶及塑膠水瓶、金屬罐頭內壁的環氧樹脂與牙齒的填充密封劑等(Krishnan et al., 1993; Brotons et al., 1995; Olea et al., 1996)

研究目的

對於台灣牙科診所使用假牙之牙材進行 BPA 溶出濃度之測定。

研究方法及材料

一、研究樣本

本研究以四種不同的假牙材料作為實驗分析，分別為 A(Alike)、REB(Rebase)、S2(Structure 2)、SOF(Soft linner)四種不同的材料，每種材料的分析數量為 18 個，共計分析 72 個樣本。

二、樣本之前處理方法參考 Theodore Eliades et al.(2007)雙酚 A(BPA)之分析方法加以研究實驗修改。將樣本溫度設定兩種 37℃、65℃再分別以 24、48、72 小時對樣本加入 15 ml 的二次水溶液、95%酒精、4%醋酸三種不同的溶液作震盪。各單獨經過 24、48、72 小時間後取出樣本將酒精水溶液以玻璃滴管取出裝入 vial 上機；另水溶液和醋酸溶液利用 5 ml 正己烷溶液做液相萃取後取出 4 ml 上清液吹氮氣至乾再回溶 2 ml 95%酒精利用玻璃滴管取出至 vial 上機。

三、分析方法

樣本經前處理後，利用高效能液相層析儀/螢光偵測器（HPLC/fluorescence detection,Hitachi Co. Japan）設定參數：

1.Column：Chromolith RP-18e（10cm × 4.6mm ID，5μm particle size）〔Merck Co.〕
2.Mobile phase：70％ acetonitrile〔Merck Co.〕 and 30％ D.I water
3.Flow-rate：1.0mL/min
4.Fluorescence detector wavelength：275 nm excitation wavelength、300 nm emission wavelength
5.Injection volume：20μL

四、品質保證(QA)與品質管制(QC)：依環保署環境檢驗所制訂之 QA/QC 規範進行。

(1)檢量線

約每一個禮拜均重新配製標準溶液，以確保標準液不因使用與存放時間過長而變質。每次檢量線每個化合物的 R 值皆＞0.9995。並設定檢量線範圍。

(2)精確度（precision）

利用添加標準品分析之樣本，以此評估樣本 BPA 之變異係數。另配製 BPA 標準溶液

(0.01、0.10、0.50、1.00 mg/L)，以 HPLC/FLD 進行分析，以計算同日和異日之變異係數(coefficient of variation, CV)。

(3)回收率之測定

利用添加 BPA 適當濃度於樣本中，經相同之樣本萃取步驟後，以 HPLC/FLD 進行分析，以此評估 BPA 測定方法之回收率。

(4)偵測極限 (detection limit)

配製儀器所能偵測出之最低濃度，將此濃度重複分析 5 次，求取平均值和標準差，以此標準差之 3 倍數值除以 5 個樣本之平均值後，再乘以所配製之濃度，即可得儀器偵測極限。

(5)查核樣本

每天樣本使用 HPLC/FLD 分析前，皆先分析 1 個中濃度檢量線的點，確定該儀器的靈敏度沒有改變。其反應面積差異在可接受範圍(5%以內)，並於每批次樣本分析後，進行查核樣本分析。

(6)避免 BPA 污染，所有玻璃器皿之清洗、試藥配製、樣本收集、保存、前處理、標準曲線製備及儀器操作，完全不使用塑膠材料於實驗過程中，完成使用玻璃器具(Pyrex Co.)，並依循標準操作程序(standard operation procedure, SOP)進行。所有程序均設法降低 BPA 之污染干擾。

結果

1.檢量線濃度範圍設定在 0.01~1.0ppm，R 值為 0.9998。BPA 檢量線如圖一。

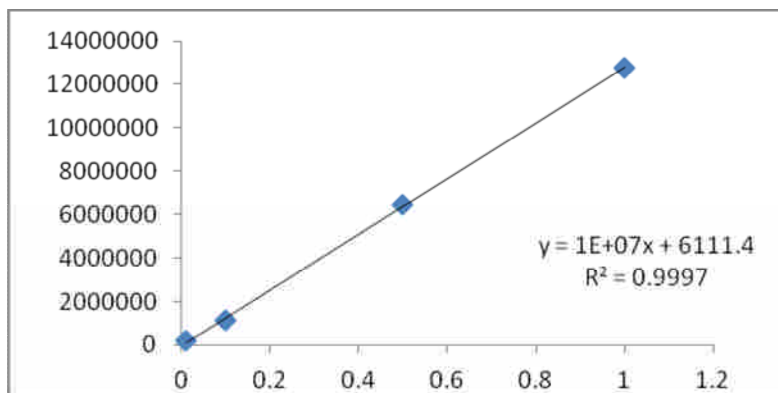
2.偵測極限：是多次重複測定檢量線最低點之水溶液後，經過計算，其方法之偵測極限為 0.5 ppb。

3. 變異係數：以低中高三個檢量線濃度值做計算，三個濃度皆重複三次做測定。
分別是 0.01、0.1、1.0 三個不同 CV 值為 0.18%、0.07%、0.08%，平均為 0.11%。CV 值百分比例如表一。

4. 回收率(Recovery)：以兩個檢量線濃度值做樣本的回收率測定，兩個濃度皆重複上機三次作檢定。分別是 0.5、1 兩個不同濃度回收率之平均值為 98.59%、97.77%。回收率之分別和平均值比例如表二。

5.樣本濃度:以 65℃各單獨震盪 24、48、72 小時酒精溶液的條件在四種牙材中溶出 BPA 之濃度為最高；其次是 37℃各單獨震盪 24、48、72 小時酒精溶液的條件在四種牙材中溶出 BPA 之濃度為最高，而四種牙材中又以 S2 溶出 BPA 濃度為最高。同時附上牙材經實驗前後的攝影照片做比較，當中發現 REB、SOF 兩種牙材會有變形之反應。各種牙材依不同時間、溫度、浸泡震盪之不同水溶液條件下的濃度值如表三、表四、表五、表六。牙材實驗前後的攝影照片如圖二、圖三、圖四、圖五、圖六、圖七。一併附上四種牙材在不同溫度中分別水溶液、酒精、醋酸的濃度趨勢圖見圖八、圖九、圖十、圖十一、圖十二、圖十三。

檢量線濃度範圍:



圖一 BPA 檢量線

變異係數：

表一、三種不同 BPA 濃度變異係數

濃度(ppm)	CV%
0.01(N=3)	0.18
0.1(N=3)	0.07
1(N=3)	0.08
Average(N=9)	0.11

回收率(Recovery)：

表二、BPA 濃度回收率

濃度(ppm)	0.5ppm	1ppm
第一次(N=3)	100.55%	100.05%
第二次(N=3)	98.08%	94.49%
第三次(N=3)	97.13%	98.78%
平均(N=9)	98.59%	97.77%

四種牙材溶出之 BPA 濃度值

表三、REB 牙材溶出濃度值

單位: ppb(W/W)

REB 牙材	65℃條件	37℃條件
24 小時-二次純水	0.008	0.013
24 小時-95 %酒精	9.637	4.426
24 小時- 4 % 醋酸	0.023	0.015
48 小時-二次純水	0.004	0.006
48 小時-95 %酒精	10.874	5.320
48 小時- 4 % 醋酸	0.015	0.019
72 小時-二次純水	0.009	0.005
72 小時-95 %酒精	11.082	7.323
72 小時- 4 % 醋酸	0.025	0.015

表四、A 牙材溶出濃度值

單位:ppb(W/W)

A 牙材	65℃條件	37℃條件
24 小時-二次純水	0.004	0.011
24 小時-95 %酒精	17.318	4.304
24 小時- 4 % 醋酸	0.046	0.014
48 小時-二次純水	ND	0.016
48 小時-95 %酒精	24.971	3.879
48 小時- 4 % 醋酸	ND	0.018
72 小時-二次純水	0.002	0.019
72 小時- 95%酒精	22.985	5.699
72 小時- 4 % 醋酸	0.017	0.016

表五、S2 牙材溶出濃度值

單位: ppb(WW)

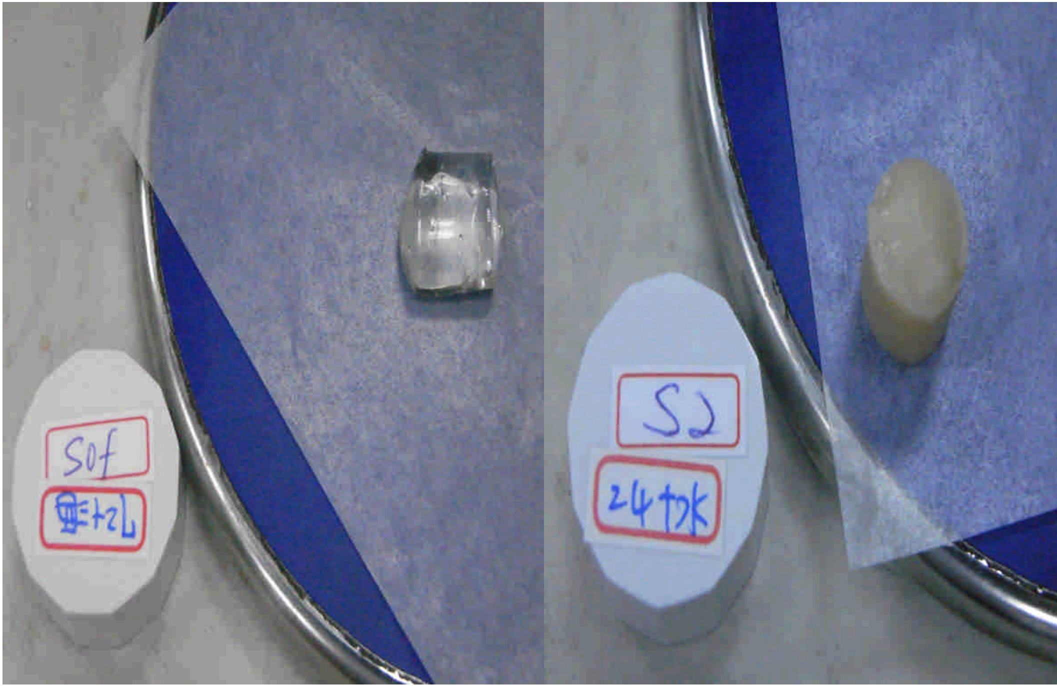
S2 牙材	65℃條件	37℃條件
24 小時-二次純水	0.759	0.334
24 小時-95 %酒精	74.702	17.853
24 小時- 4 % 醋酸	0.361	0.171
48 小時-二次純水	1.015	0.249
48 小時-95 %酒精	87.902	20.164
48 小時- 4 % 醋酸	0.670	0.134
72 小時-二次純水	1.600	0.610
72 小時- 95 %酒精	80.494	29.824
72 小時- 4 % 醋酸	0.839	0.127

表六、SOF 牙材溶出濃度值

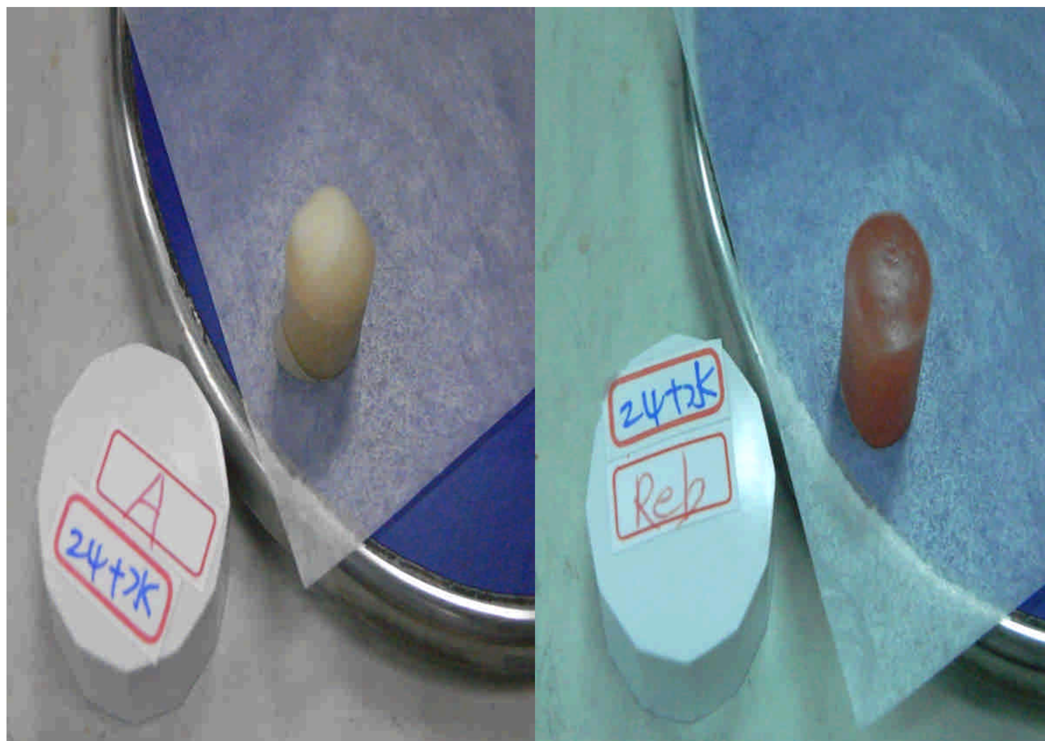
單位: ppb(W/W)

SOF 牙材	65℃條件	37℃條件
24 小時-二次純水	0.011	0.011
24 小時-95 %酒精	23.445	3.559
24 小時- 4 % 醋酸	0.016	0.016
48 小時-二次純水	0.011	0.012
48 小時-95 %酒精	24.301	4.427
48 小時- 4 % 醋酸	ND	0.017
72 小時-二次純水	0.016	0.012
72 小時-95 %酒精	44.391	4.967
72 小時- 4 % 醋酸	0.359	0.017

牙材經實驗前後之攝影照片:



圖二 (左、SOF 牙材未經實驗處理前照片；右、S2 牙材未經實驗處理前照片)

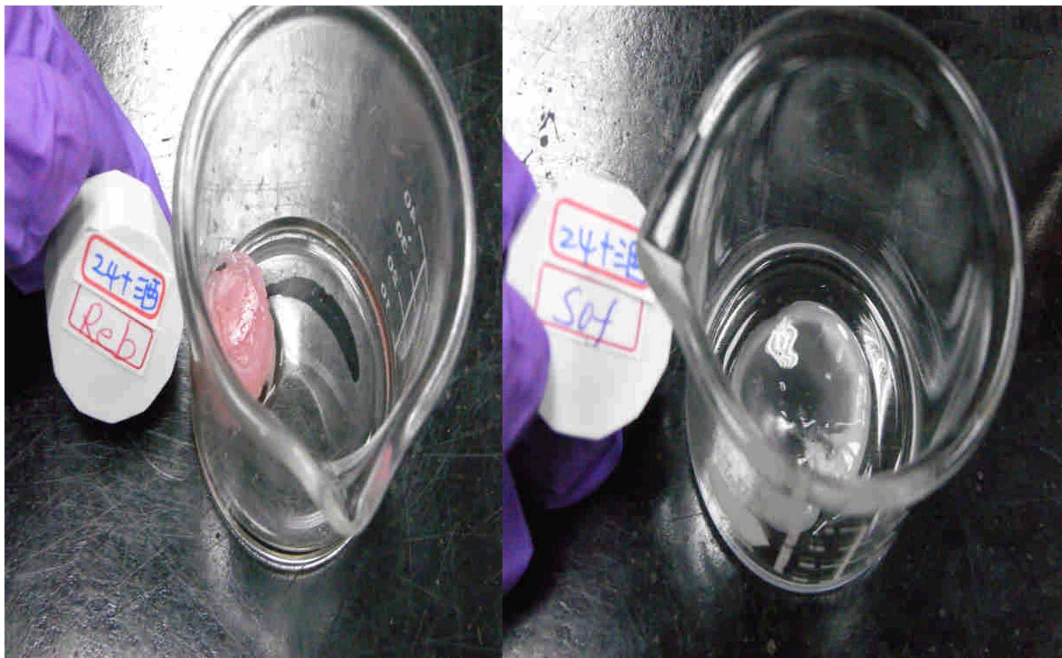


圖三 (左、A 牙材未經實驗處理前照片；右、REB 牙材未經實驗處理前照片)

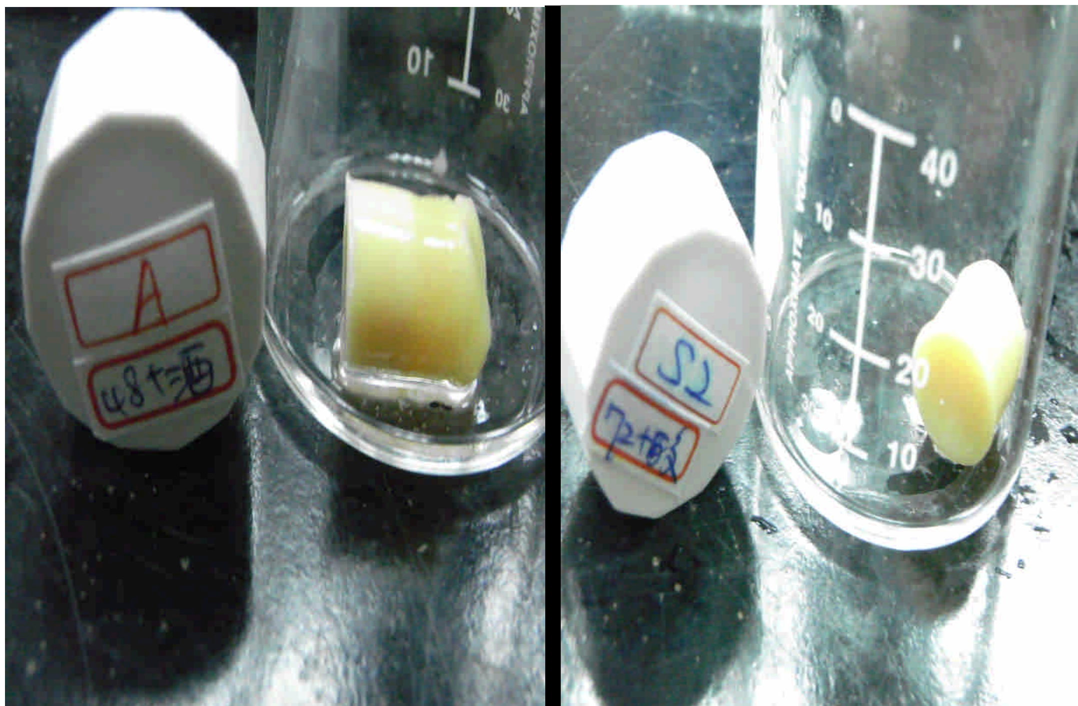
經實驗處理後之攝影:

圖四(SOF 牙材經 65℃(24、48、72 小時)酒精處理後呈現透明帶白色圓餅狀)

圖五(REB 牙材經 65℃(24、48、72 小時)酒精處理後呈現粉紅色膨脹軟狀易散開)

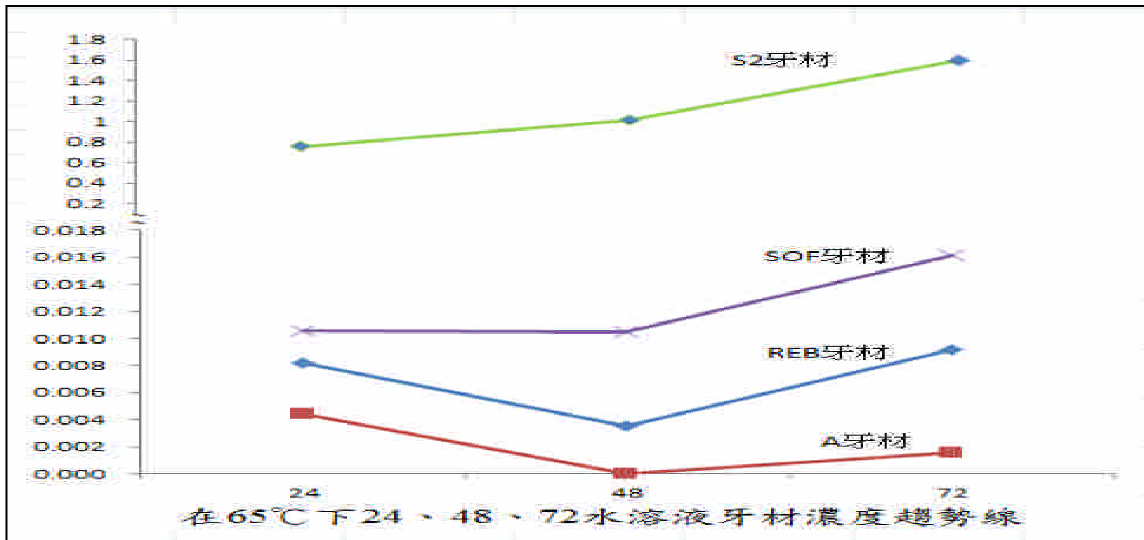


圖六(左、REB 牙材經 37℃(24、48、72 小時)酒精處理後呈現粉紅色膨脹軟狀易散開；右、SOF 牙材經 37℃(24、48、72 小時)酒精處理後呈現透明帶白色圓餅狀)

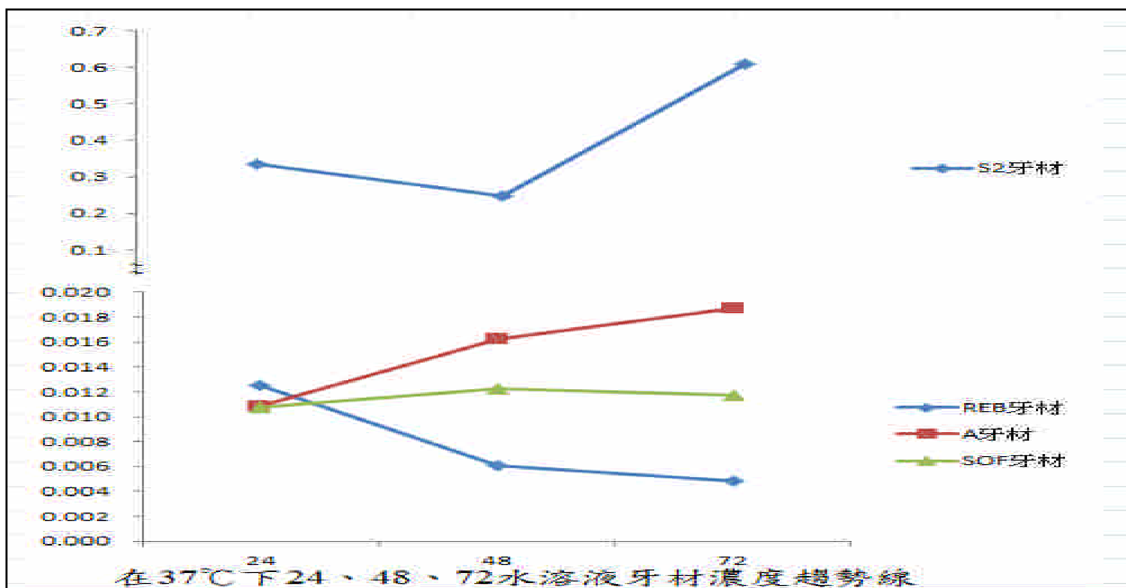


圖七(A 牙材與 S2 牙材經 37℃與 65℃(24、48、72 小時)水、酒精、醋酸實驗後外型不會造成改變)

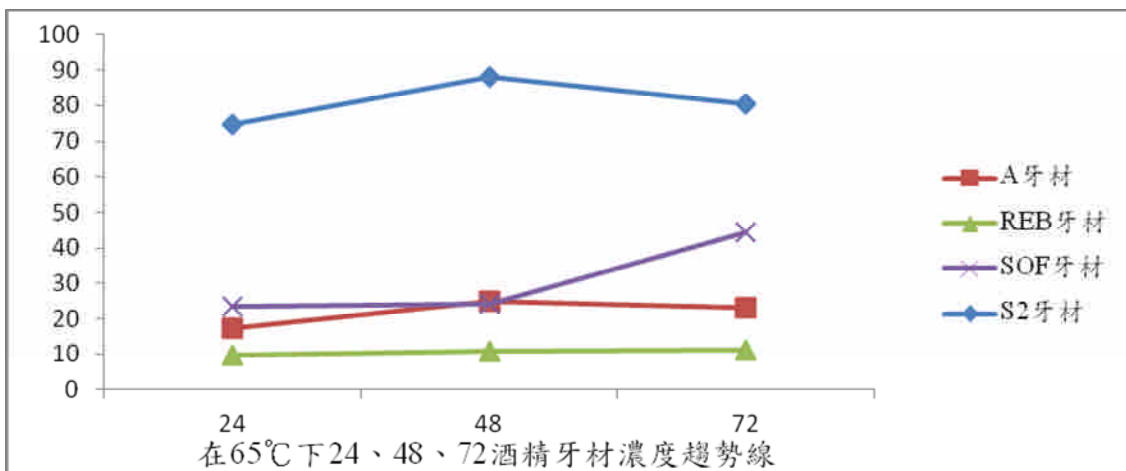
牙材之濃度趨勢線:



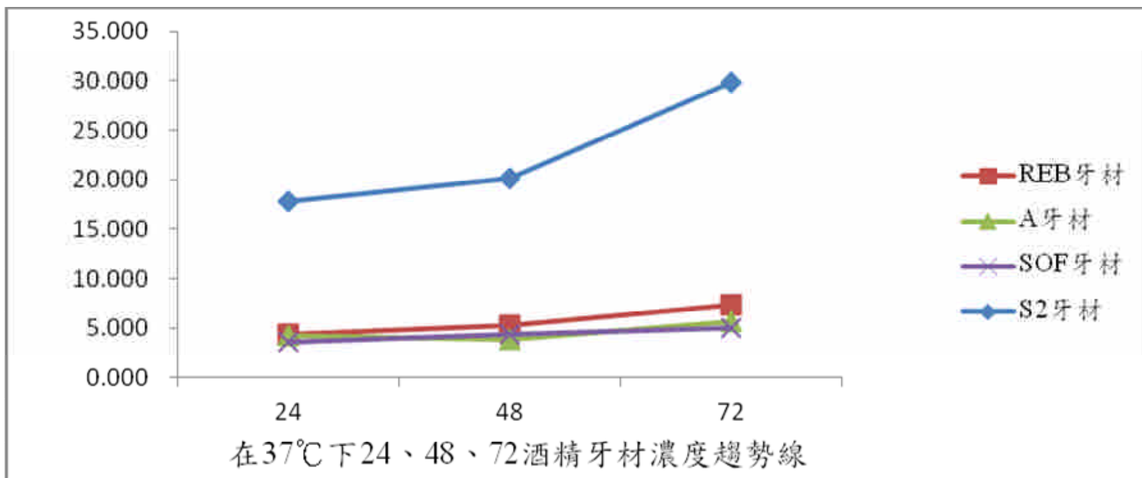
圖八、65℃下水溶液濃度趨勢



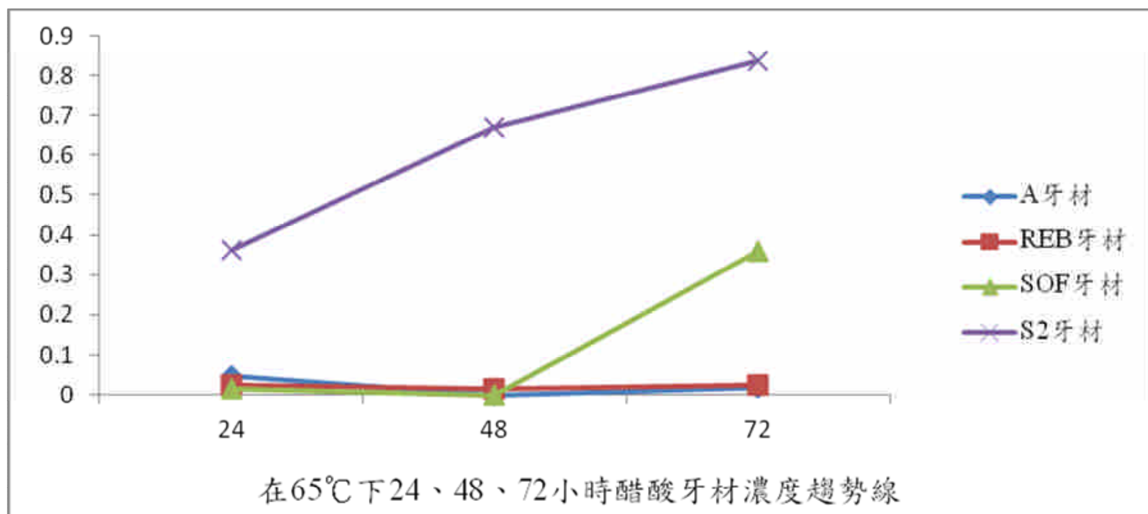
圖九、37℃下水溶液濃度趨勢



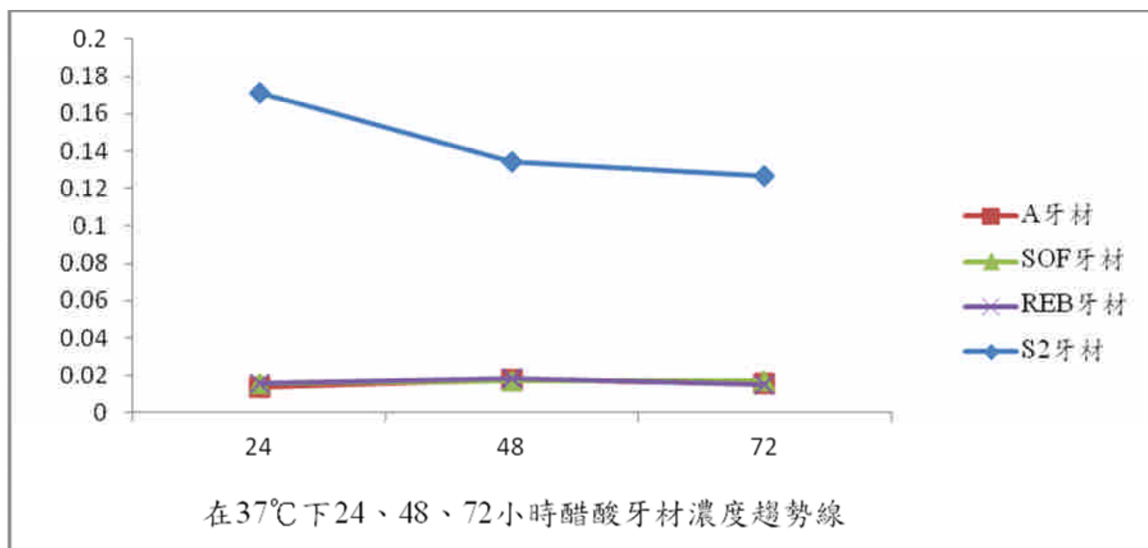
圖十、65℃下酒精溶液濃度趨勢



圖十一、37℃下水溶液濃度趨勢



圖十二、65℃下醋酸溶液濃度趨勢



圖十三、37℃下醋酸溶液濃度趨勢

結論

一、 本研究分析四種不同材質的假牙牙材。依樣本乾重計，共計 72 個樣本。分析之所有四種樣本中之 BPA 濃度以 S2 牙材中 65℃在酒精中震盪 48 小時的條件為最高，其次以 S2 牙材中 65℃在酒精溶液中震盪 72 小時的條件為次高，濃度為 87.902ppb 以及

80.494ppb；在 37℃中之 BPA 濃度以 S2 牙材中震盪 72 小時的酒精為最高，濃度為 29.824ppb，其次分別是 S2 牙材 48 小時酒精及 S2 牙材 24 小時酒精，濃度分別為 20.164ppb 及 17.853ppb。

二、 由於分析不同四個牙材，可以得到實驗結果證明暴露濃度在相同條件下相當一致，在不同的條件下牙材暴露到的濃度值會隨著時間增加。

三、 發現其實驗前後 SOF 及 REB 在酒精的條件下不論是 37℃或 65℃震盪 24、48、72 小時牙材材質皆會變形；而 S2 和 A 則沒有發現改變，但 A 牙材的液體會呈現透明帶黃色狀，S2 則是透明澄清狀。

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

100 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
會議 時間 地點	100 年 3 月 16 日至 3 月 19 日 美國，聖地牙哥（San Diego,California）	本會核定補助文號	NSC 99-2314-B-040-015
會議名稱	（中文）第 89 屆國際牙醫研究學會年會 （英文）89th General Session & Exhibition of the international Association for Dental Research		
發表論文題目	（中文）血小板纖維蛋白對人牙周膜纖維細胞的影響 （英文）Effects of platelet-rich fibrin on human periodontal ligament fibroblasts.		

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美國聖地牙哥舉行，距離 2002 年 IADR 會議已有九年，聖地牙也改變很多，變得人口更多、更為繁榮飯店餐館賣場林立，觀光人潮不斷湧進，讓很多參加牙醫研究學會會員留下深刻映像，尤其是可利用這次機會參觀美國哥航空母艦更是不虛此行。

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“Effects of platelet-rich fibrin on human periodontal ligament fibroblasts”以 poster presentation 方式發表。研究血小板纖維蛋白對人牙周膜纖維細胞的影響，血小板纖維蛋白(Platelet-rich fibrin ；PRF) 是由法國 Choukroun 醫師所創，抽血後離心所取得中段的血小板凝膠，可以用來混合人工骨粉形成魚漿狀容易操作，更可以加速傷口癒合,國外的醫學報告證實可以產生較多的自體骨,而且讓傷口幾乎不痛， PRF 血小板凝膠是網狀纖維結構，內含生長因子，可以加速骨細胞附著生長,也可加速傷口癒合，高濃度

的生長因子均勻附著在網狀纖維中，隨著網狀纖維分解而釋出，可以延長生長因子的作用時效。

二、與會心得

本次我們組團，團員中國、中山與高雄等三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。

接受函
IADR/AADR 2011 San Diego - Poster Acceptance Notification
December 10, 2010
Fu-Mei Huang
Chung Shan Medical University
Taichung R.O.C, Taiwan
Dear Fu-Mei Huang, Abstract ID#144856:

It is a pleasure to inform you that your abstract has been ACCEPTED as a POSTER PRESENTATION at the IADR/AADR/CADR 89th General Session and Exhibition (March 16-19, 2011). The meeting will take place at the San Diego Convention Center in San Diego, California.

Please note that some students/co-workers have provided an alternate e-mail address for notification, so if this letter is addressed to a colleague, please forward it to his/her attention. E-mail notifications are sent only to the address provided for the presenter when the abstract was submitted; it is the presenter뭑 responsibility to notify co-authors.

The following is the notification of the session to which your paper has been assigned. Please DO NOT lose this notification. The mode of your presentation has been assigned by the Annual Session Committee and Group Program Chair and must be followed as we are unable to change it at this date. Assignments were based on authors?requests as much as possible. Please note that in contrast to previous years, your final presentation number will be assigned next month (see note below for details).

PRESENTATION INFORMATION

Presentation Date: Saturday, March 19, 2011 Session Title: Wound Healing and Regeneration, In Vitro Studies Session Time: 1:45 p.m.
San Diego Convention Center: Hall C
Schedule to follow if presenting on Thursday or Friday:
Poster Viewing Time: 8 a.m. ?5 p.m.
Poster Set-up Time: 7:30 a.m. ?8 a.m.
Poster Tear-down Time: 5 p.m. ?5:15 pm

Schedule to follow if presenting on Saturday:
Poster Viewing Time: 8 a.m. ?3 p.m.
Poster Set-up Time: 7:30 a.m. ?8 a.m.
Poster Tear-down Time: 3 p.m. ?3:15 p.m.

POSTER SIZE
The size for your poster board will be 4' high x 8' wide so the board will be used HORIZONTALLY. These are the maximum dimensions to follow when creating your poster but you may make your poster smaller. You are only required to be at your poster board during the session time listed and not the entire poster viewing time. For further information please go directly to the meeting page on the IADR website (see the link below).

POSTER SUPPLIES
Please make every attempt to bring your own pushpins or thumbtacks to mount your poster as a limited supply will be available.

PRE-REGISTRATION REQUIREMENT
All presenters must pre-register and pay the applicable fee by the January 12, 2011 presenter deadline. Approximately one week after the presenter registration deadline, you will be sent a notification confirming your participation with your final presentation and session sequence numbers (the notices will be sent to all presenters at once after the registration deadline and not sent individually after you register). If you do not pre-register, you will NOT be allowed to participate in the meeting and your abstract will be withdrawn from the final printed Program Book and will not be citable as appearing in the special edition of the Journal for Dental Research. If you need an invitation letter to get a visa to enter the United States, please check the applicable box when registering and a letter will be sent to you via e-mail.

IMPORTANT MEETING LINKS

Program Book Listing:
Your title will be printed in the Program Book as submitted here:
Effects of Platelet-rich Fibrin on Human Periodontal Ligament Fibroblasts
If you would like to edit your presentation title (not to exceed 10 words), please visit the link below by January 3, 2011 and use your assigned Abstract ID 144856 and password 125965:
http://iadr.confex.com/iadr/2011sandiego/prt/extra/index.cgi?
Presenter Guidelines:
http://www.aadronline.org/i4a/pages/index.cfm?pageid=3760
Online Scientific Program:
http://iadr.confex.com/iadr/2011sandiego/webprogram/
To register for the meeting (click the "Register Online" link):
http://www.iadr.org/iags
You will need your Abstract ID# to register for the meeting: 144856
To reserve a hotel room (click the "Reserve your hotel online" link):
http://www.iadr.org/iags
General Meeting Information:
http://www.iadr.org/iags
If you have arranged your travel already and you arrive later or depart earlier than your assigned presentation time, we will not be able to move your presentation to accommodate your travel plans.

Thank you for submitting your paper. We look forward to your presentation at the meeting and we have scheduled a full conference so we hope you stay for the duration of the meeting. If you have any questions, please send a message to meetings@iadr.org. Every attempt will be made to respond as soon as possible.
Sincerely,
E. Dianne Rekow, DDS, PhD
IADR Annual Session Committee Chair
Jeffrey L. Ebersole, PhD
AADR Annual Session Committee Chair

全文與摘要

144856 Effects of Platelet-rich Fibrin on Human Periodontal Ligament Fibroblasts

Saturday, March 19, 2011: 1:45 p.m. - 3 p.m.

Location: Hall C (San Diego Convention Center)

Presentation Type: Poster Session

F.-M. HUANG, *J. ZHAO, and Y.-C. CHANG, School of Dentistry, College of Oral Medicine, Chung Shan Medical University, Taichung R.O.C, Taiwan*

Objectives: Platelet-rich fibrin (PRF) by Choukroun's technique is derived from an autogenous preparation of concentrated platelets without any manipulation and is widely used in implant dentistry. Recently, PRF was reported to increase periodontal ligament fibroblasts (PDLFs) proliferation (J Dent Sci 2009; 4: 130-5). However, the underlying mechanisms are not yet completely understood. This study was to determine the effects of PRF on the expression of phosphorylated extracellular signal-regulated protein kinase (p-ERK), osteoprotegerin (OPG), and alkaline phosphatase (ALP) on PDLFs. Methods: Blood collection was carried out from ten healthy volunteers. The PRF samples were obtained from PC-02 table centrifuge centrifuged at 3000 rpm for 12 min. Human PDLFs were derived from two healthy individuals undergoing extraction for orthodontic reasons with informed consents. Western blot was employed to evaluate the expression of p-ERK and OPG in PDLFs by PRF stimulation. ALP activity was examined by substrate assay. Results: PRF was found to increase ERK phosphorylation in PDLFs in a time-dependent manner ($p<0.05$). OPG was also significantly elevated by the stimulation with PRF ($p<0.05$). In addition, ALP activity was significantly upregulated by PRF ($p<0.05$). Conclusions: Taken together, the activation of p-ERK, OPG, and ALP expression by PRF suggests a potential role for hard tissue engineering. The application of PRF may provide the benefit for the periodontal regeneration.

Keywords: Fibroblasts, Inflammation, Periodontics and Periodontium-gingiva

Effects of platelet-rich fibrin on human periodontal ligament fibroblasts.

FM Huang*, Zhao JH, and YC Chang (School of Dentistry, Chung Shan Medical University, Taichung, Taiwan)

Objectives: Platelet-rich fibrin (PRF) by Choukroun's technique is derived from an autogenous preparation of concentrated platelets without any manipulation and is

widely used in implant dentistry. Recently, PRF was reported to increase periodontal ligament fibroblasts (PDLFs) proliferation (J Dent Sci 2009; 4: 130-5). However, the underlying mechanisms are not yet completely understood. This study was to determine the effects of PRF on the expression of phosphorylated extracellular signal-regulated protein kinase (p-ERK), osteoprotegerin (OPG), and alkaline phosphatase (ALP) on PDLFs. **Methods**: Blood collection was carried out from ten healthy volunteers. The PRF samples were obtained from PC-02 table centrifuge centrifuged at 3000 rpm for 12 min. Human PDLFs were derived from two healthy individuals undergoing extraction for orthodontic reasons with informed consents. Western blot was employed to evaluate the expression of p-ERK and OPG in PDLFs by PRF stimulation. ALP activity was examined by substrate assay. **Results**: PRF was found to increase ERK phosphorylation in PDLFs in a time-dependent manner ($p<0.05$). OPG was also significantly elevated by the stimulation with PRF ($p<0.05$). In addition, ALP activity was significantly upregulated by PRF ($p<0.05$). **Conclusions**: Taken together, the activation of p-ERK, OPG, and ALP expression by PRF suggests a potential role for hard tissue engineering. The application of PRF may provide the benefit for the periodontal regeneration.

Introduction

Platelet-rich fibrin (PRF) described by Choukroun *et al*. (2000) is a second-generation platelet concentrate which allows one to obtain fibrin membranes enriched with platelets and growth factors, after starting from an anticoagulant-free blood harvest (Dohan *et al*, 2006a, 2006b). PRF looks like a fibrin network and leads to more-efficient cell migration and proliferation and thus cicatrization (Dohan Ehrenfest *et al*, 2010). This unique structure may be capable of acting as a vehicle for carrying of cells that are essential for tissue regeneration. In addition, the PRF membrane could serve as a resorbable membrane for guided tissue regeneration.

Many growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β, are released from PRF (Dohan *et al*, 2006a, 2006b, Dohan Ehrenfest *et al*, 2009). Recently, studies have demonstrated that the PRF membrane has a very significant slow sustained release of key growth factors for at least 1 week (Dohan *et al*, 2006b) and up to 28 days (He *et al*, 2009), which means that the membrane stimulates its environment for a significant time during wound healing. Some clinical applications have been described in oral surgery (Choukroun *et al*, 2006a, 2006b) and dental implantology (Diss *et al*, 2008, Mazor *et al*, 2009). However, there are few references in the literature about the biological properties of PRF for periodontal regeneration.

One of the most dominant factors in the regeneration of periodontal tissues is periodontal ligament cells. Human periodontal ligament fibroblasts (PDLFs), which play an important role in alveolar bone formation and resorption in the development of periodontitis, form a heterogenous population, with some cells having osteoblast-like characteristics and the potential to differentiate into osteoblasts (Basdra *et al*, 1997). There is also strong alkaline phosphatase (ALP) activity in PDLFs, which appears to be quite important for the apposition of acellular cementum

(Beertsen *et al*, 1997).

Recently, PRF was found to stimulate human PDLFs proliferation (Tsai *et al*, 2009). However, the underlying mechanisms are not yet completely understood. The extracellular signal-regulated protein kinase (ERK) signaling pathway is one of the mitogen-activated protein kinase cascades and plays important roles in the regulation of cell growth and differentiation (Segar 1995). Osteoprotegerin (OPG), a naturally occurring inhibitor of osteoclast differentiation, binds to receptor activator of nuclear factor-κB ligand (RANKL) and blocks RANKL from interacting with RANK (Lacey *et al*, 1998). Alkaline phosphatase (ALP) is one of the markers of osteoblastic differentiation. The purpose of this study was to determine whether PRF could influence the functions of PDLFs. The effects of PRF on human PDLFs were determined through measuring protein expression of p-ERK, OPG, and ALP.

Materials and methods

PRF preparation

The human blood samples in this study were obtained under the permission of Institutional Review Board at Chung Shan Medical University Hospital. Blood from 10 non-smoking healthy volunteers were treated according to the PRF protocol (Mazor *et al*, 2009) with a PC-02 table centrifuge and collection kits provided by Process (Nice, France). Briefly, blood samples were taken without an anticoagulant in 10 ml glass-coated plastic tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ, USA) and immediately centrifuged at 3,000 rpm for 10 min. A fibrin clot formed in the middle part of the tube, while the upper part contained acellular plasma, and the bottom part contained red corpuscles. The fibrin clot was easily separated from the lower part of the centrifuged blood. The PRF clot was gently pressed into a membrane with sterile dry gauze. From hematoxylin and eosin-stained section, PRF demonstrated a membrane-like appearance. Homogenous and intensive staining of fibrin matrix was found. Leucocytes were located within the PRF membrane. For scanning electron microscopy (SEM) observation, the PRF membrane surface showed the print of the gauze threads. Occasionally, platelet aggregates could be found on the surface.

Cell cultures

After approval by the Hospital Review Board, fibroblasts were obtained from the periodontal ligament of premolar teeth from two patients undergoing extraction for orthodontic reasons as described previously (Chang *et al*, 2001, Chang *et al*, 2003). After extraction, the teeth were placed in Petri dishes containing Dulbecco's modified Eagle's medium (DMEM) (GIBCO; Grand Island, NY, USA) and 100 units of penicillin and 100 μg of streptomycin/ml. To avoid contamination from gingiva, the periodontal ligament was carefully removed from the middle third of the root by

scalpel. The fragments were grown in DMEM supplemented with 10 % fetal calf serum (FCS) and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 and 95 % air. Confluent cell layers were treated with 0.25 % trypsin and 0.05 % EDTA for 5 min; aliquots of separated cells were subcultured and then used between the 3rd to 8th passages.

Western blot for p-ERK and OPG

Confluent PDLFs were trypsinized, counted, and plated at a concentration of 5×10^4 cells in 60 mm culture dish and allowed to achieve confluence. Nearly confluent monolayers of cells were washed with serum free DMEM for 24 h and immediately thereafter exposed to PRF for the indicated times. Cell lyates were collected on day 1, 3, and 5 as described previously (Tsai *et al*, 2009). Briefly, cells were solubilized with SDS-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl , pH 7.5 and 0.5 % Trition X-100, 2 mM phenylmethysulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12,000g at 4°C and the protein concentrations determined with Bradford reagent using bovine serum albumin (BSA) as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10 % SDS-PAGE and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3 % BSA for 2 h, rinsed, and then incubated with primary antibodies, anti-p-ERK (Cell Signaling Technolnogy, Beverly, MA,USA) (1:1000) or anti-OPG (Abcam, Cambridge, MA,USA) (1:1000) in PBS containing 0.05 % Tween 20 for 2 h. After 3 washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above and treated with 1:1000 streptavidin-peroxidase solution for 30 min. After a series of washing steps, the reactions were developed using diaminobenzidine (Zymed, South San Francisco, CA, USA). The intensities of the obtained bands were

determined using a densitometer (AlphaImager 2000). Each densitometric value was expressed as the mean ± SD.

Measurement of ALP activity

The cell lyates were sonicated on ice bath, centrifuge 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 PDLFs 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. Statistical analysis was by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test.

Results

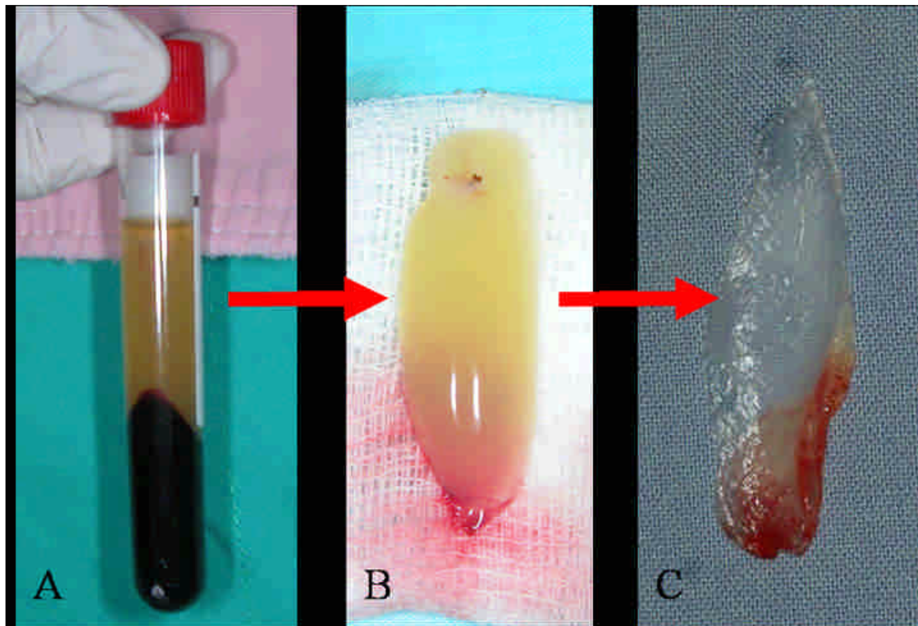


Fig. 1 (a) PRF formed in the middle part of the tube. The upper part contained acellular plasma, and the bottom part contained red corpuscles. (b) The fibrin clot was easily separated from the lower part of the centrifuged blood. (c) The PRF clot was gently pressed between 2 layers of sterile dry gauze to form a membrane.

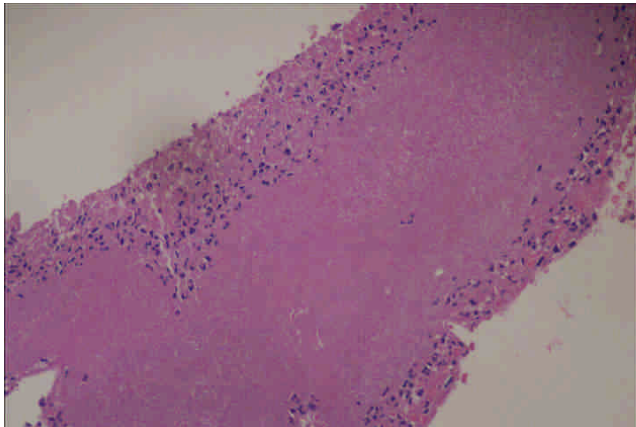


Fig. 2 (a) Light microscope examination of PRF reveals a homogenous fibrin appearance by hematoxylin and eosin stain. Leucocytes were located within the PRF matrix. (original magnification x100)

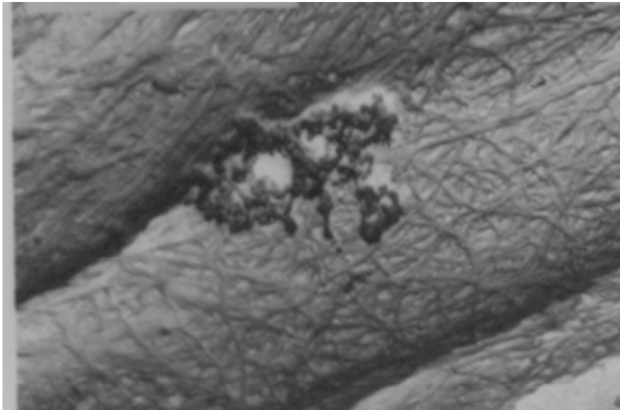


Fig. 2 (b) SEM image of PRF membrane showed the print of the gauze threads that were used for the final PRF clot compression into a membrane before fixation for histologic analysis. Platelet aggregates are assembled on the surface occasionally. (original magnification x3500)

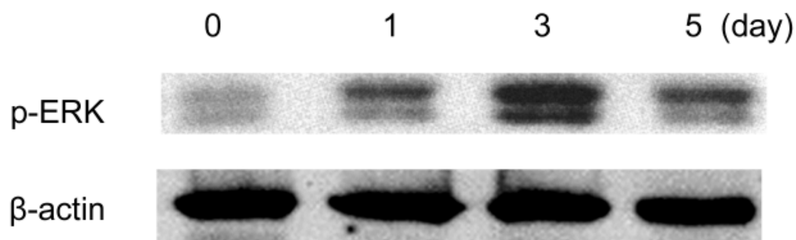


Fig. 3 (a) Kinetics of p-ERK expression in PDLFs exposed to PRF for 1, 3, and 5 days, respectively by Western blot analysis. β-actin was performed in order to monitor equal protein loading.

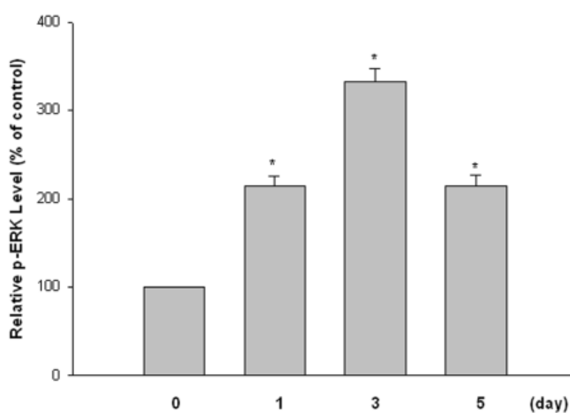


Fig. 3 (b) Levels of p-ERK protein treated with PRF were measured by AlphaImager 2000. The relative level of p-ERK protein expression was normalized against β-actin signal and the control was set as 1.0. Optical density values represent the mean ± SD. * represents significant difference from control values with $p<0.05$.

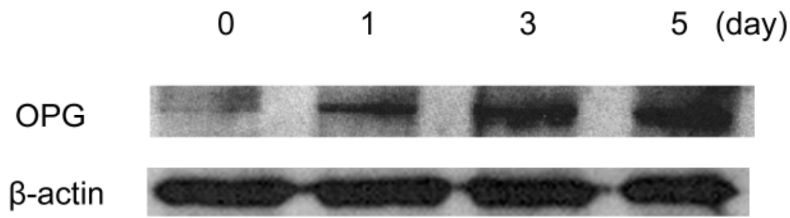


Fig. 4 (a) Kinetics of OPG expression in PDLFs exposed to PRF for 1, 3, and 5 days, respectively by Western blot analysis. β-actin was performed in order to monitor equal protein loading.

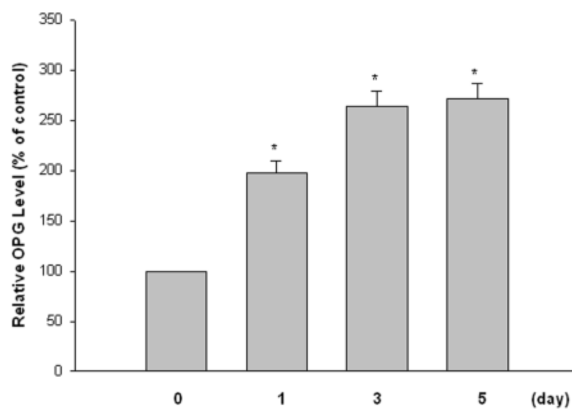


Fig. 4 (b) Levels of OPG protein treated with PRF were measured by AlphaImager 2000. The relative level of OPG protein expression was normalized against β-actin signal and the control was set as 1.0. Optical density values represent the mean ± SD. * represents significant difference from control values with $p<0.05$.

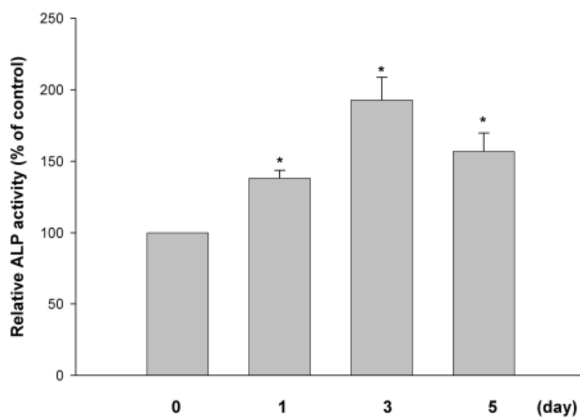


Fig. 5 Kinetics of ALP activity in PDLFs exposed to PRF for 1, 3, and 5 days,

respectively by substrate assay. The percentages of ALP activity in the presence of PRF relative to that in the control are shown. The results were averaged from three independent experiments. * denotes significant differences from control values with $p<0.05$.

Conclusion

Choukroun's PRF is derived from an autogenous preparation of concentrated platelets without any manipulation and is widely used in implant dentistry as a vector for cell growth factors. In this study, PRF was demonstrated to enhance p-ERK, OPG, and ALP expression. Using PRF may provide the potential benefits in periodontal regeneration. Future studies are required to elucidate the precise mechanism of action of PRF in the complicated network of various cell types in the periodontal regeneration.

國科會補助計畫衍生研發成果推廣資料表

日期:2011/10/29

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<tr><td>計畫主持人: 黃富美</td></tr>
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99 年度專題研究計畫研究成果彙整表

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計畫名稱：研究牙科樹脂牙冠中雙酚 A 的釋出量							
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	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
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		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	1	100%		
		專書	0	0	100%	章/本	
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		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

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<tr><td>舉辦之活動/競賽</td><td>0</td><td></td></tr>
<tr><td>研討會/工作坊</td><td>0</td><td></td></tr>
<tr><td>電子報、網站</td><td>0</td><td></td></tr>
<tr><td>計畫成果推廣之參與（閱聽）人數</td><td>0</td><td></td></tr>
</table>

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，作一综合評估。

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综合評估 ■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）