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計畫編號：DOH88-HR-815

行政院衛生署八十八年度委託研究計畫

靈芝子實體作為皮膚取代物之探討研究

委託研究成果報告

執 行 機 構：私立臺北醫學院

計 畫 主 持 人：蘇慶華

執 行 期 限：87年7月1日至88年6月30日

＊＊本研究報告僅供參考用，不代表本署意見＊＊

計畫編號：DOH88-HR-815

行政院衛生署八十八年度整合性醫藥衛生科技研究計畫

靈芝子實體作爲皮膚取代物之探討研究

年度成果報告

執 行 機 構：私立臺北醫學院

計 畫 主 持 人：蘇慶華

執 行 期 間： 87 年 7 月 1 日 至 90 年 6 月 30 日

表一 、傷口面積變化

單位：cm^2

	第 4 日*		第 8 日*		第 12 日*		第 16 日*		第 20 日*	
編號	靈芝	紗布	靈芝	紗布	靈芝	紗布	靈芝	紗布	靈芝	紗布
1	4.42	4.50	2.77	2.74	1.69	1.89	0.65	1.19	0.22	0.87
2	4.62	5.01	2.89	3.50	1.47	1.79	0.89	1.25	0.19	0.59
3	4.92	5.12	2.97	3.25	1.48	2.01	1.01	1.43	0.25	0.96
4	4.04	4.80	2.97	3.31	2.32	3.04	0.98	2.11	0.21	1.25
5	4.24	5.19	3.05	3.42	1.96	2.32	0.78	1.30	0.17	0.81
6	4.15	4.46	2.87	3.10	1.57	2.26	1.02	1.37	0.30	0.68
7	4.08	5.01	3.10	3.81	2.04	2.20	0.81	1.07	0.22	0.86
8	4.22	4.34	3.05	3.38	1.48	2.12	0.65	1.10	0.32	0.63

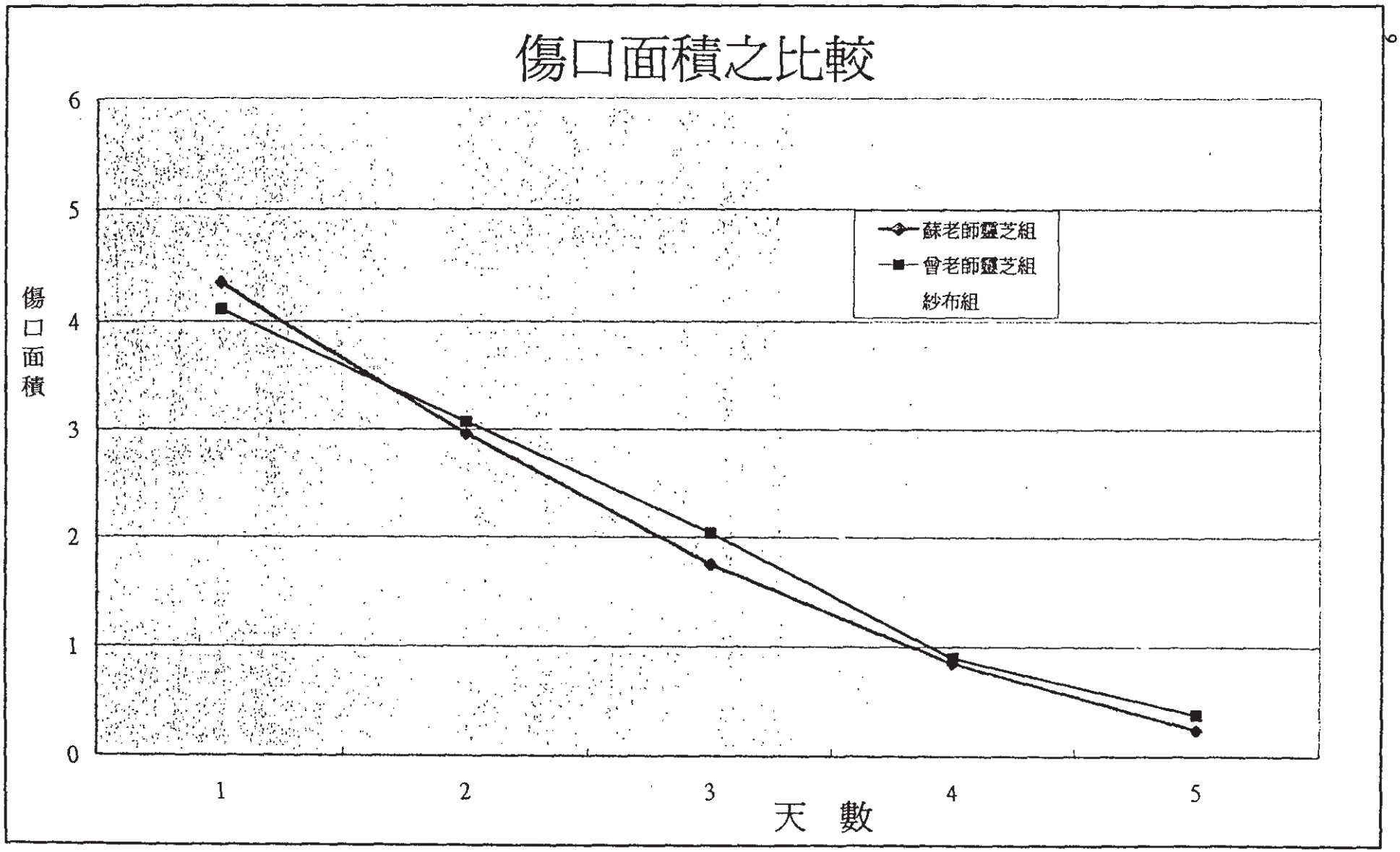
* 經單尾 Paired T test 檢定有顯著差別。

表二、傷口面積變化

單位：cm^2

	第 4 日*		第 8 日*		第 12 日*		第 16 日*		第 20 日*	
編號	靈芝	紗布	靈芝	紗布	靈芝	紗布	靈芝	紗布	靈芝	紗布
1	4.37	4.53	3.32	3.90	2.01	2.32	1.02	1.90	0.63	0.72
2	4.33	4.60	3.05	3.81	1.87	2.25	0.98	1.22	0.52	0.70
3	3.78	4.25	2.95	3.41	2.29	2.50	1.08	1.87	0.33	0.66
4	3.91	4.24	2.98	3.50	1.90	2.25	0.53	1.61	0.37	0.51
5	4.09	5.01	3.05	3.88	1.95	2.08	0.57	1.94	0.19	1.01
6	4.11	4.34	3.16	3.72	2.12	2.61	1.05	1.31	0.23	0.90
7	4.20	4.45	3.14	3.40	2.17	3.00	0.88	1.31	0.22	0.63
8	4.12	4.37	2.89	3.09	1.98	2.51	1.10	1.88	0.57	0.95

* 經單尾 Paired T test 檢定有顯著差別。


傷口面積之比較
傷口面積
6
5
4
3
2
1
0
1
2
3
4
5
天 數
蘇老師靈芝組
曾老師靈芝組
紗布組

子計劃-2（胡俊弘）

摘要

本子計劃到本年度，繼續測驗大鼠對於不同處理方式所得到之靈芝膜進行癒傷促進，靈芝薄膜計有兩種不同製造方式，第一種製造方式取靈芝廢梓以酒精去除殘餘之油溶性物質，再以 1N NaOH 於 100℃ 下處理 2-3 小時後，清洗鹼液後以適當次氯酸鈉溶液(1%)進行漂白去除 melamin 等色素後，清洗次氯酸鈉而以過濾方式，得到靈芝紙漿，經轉模，急速冷凍成型後，以冷凍乾燥固定成型，其成薄的方式是以菌絲與菌絲表面之間以氫鍵方式互相結合而成。其優點在於冷凍過程中形成冰晶，經冷凍乾燥後留下孔隙，對於癒傷過程中透氣性較佳，缺點是遇到傷口之體液後，結構會散失。

第二種成膜方式，是以靈芝癈梓經酒精處理後，以 LiCl/MeOH 溶劑溶解幾丁質後去除不溶物，次溶解物逐漸復水，使薄膜沉澱成形，形成分子與分子間以氫鍵結合，於傷口使用時不會崩解，但其缺點爲透氣性較差。

此兩種癒傷過程，促進傷口癒合速度均較控制組(紗布)顯著縮短癒合時間，而兩種處理以菌絲結合的方式稍佳但差異不顯著。

子計畫-3（蔡郁惠）

摘要

目前有許多的生物醫學材料已被運用在醫療用途上，而靈芝(*Ganoderma tsugae*)就是屬於這些生物材料中的一種。靈芝的成份已被研究出具有很多的效用，如：治療癌症，降低血壓、血醣等功能。而且目前並沒有任何報告指出他對於人體有所傷害，因此它是一種非常安全的生物材料。最近實驗證明，由靈芝子實體所製成的靈芝皮(SACCHACHITIN)與一般的紗布比較，有縮短老鼠傷口的癒合時間的現象，所以靈芝皮應可運用在人類的受傷皮膚上，作一種有效的人工皮膚取代物。

在臨床應用之前，我們必需先瞭解靈芝皮液對於動物體纖維母細胞是否具有毒性反應，以及直接觀察老鼠對於靈芝皮的過敏反應，並利用酵素連結免疫螢光分析法(ELISA)來研究靈芝皮是否對老鼠會產生抗原抗體的反應。

我們的研究成果顯示，靈芝皮及靈芝膜加幾丁質以及純靈芝膜對於纖維母細胞在小於 0.05%的濃度下並無毒性的現象發生。另外，我們利用皮下注射方式給予老鼠靈芝皮液，除了在注射點有隆起外，並無發紅的過敏現象產生。另外，這些打入靈芝皮液的老鼠血清、與注射 PBS 的老鼠血清及注射豬的 collagen type I 或 collagen type II 的老鼠血清相比，並沒有明顯的抗原抗體反應發生，雖然豬的 collagen type I 及 collagen type II 皆在老鼠血清內呈現抗體反應。依據我們的實驗結果可以知道靈芝皮對於老鼠並無傷害。另外，在組織學上發現在傷口癒合的第一天有覆蓋靈芝皮的比覆蓋紗布的傷口其

neutrophils 出現的多，在第七天則雖兩者都有痂皮(scar)組織的形成，也皆有角質細胞爬向傷口處，但是在覆蓋靈芝皮的傷口上比覆蓋紗布的傷口其角質細胞層的生長與爬動多很多。

關鍵字：靈芝皮，皮膚傷口癒合，細胞毒性，免疫性，角質細胞。

子計畫-4（曾厚）

摘要

靈芝已經是一種重要的生物醫學材料，而且近年來不論在生物學上之應用或在作爲健康食物等都已被廣泛地探討了。一般，靈芝在經水淬取後會殘留90%以上的菌絲纖維被廢棄。而這些殘餘的菌絲纖維其化學性質與殼糖(chitin)相似，因此本子計劃的主要目的是利用殼糖、纖維素(cellulose)及尼龍(nylon)等物質的溶解機構爲基礎設計出可溶解菌絲纖維的溶劑系統，進行系列研究。因爲溶劑二甲基甲醯胺中的氯化鋰的濃度會影響菌絲纖維的溶解度，因此首先，探討不同氯化鋰濃度在二甲基甲醯胺中的變化，發現二甲基甲醯胺中最大的溶解度約爲8%。且也將所得溶液以毛細管粘度測定儀測定二者間的濃度粘度函數。接著以不同濃度的氯化鋰與二甲基甲醯胺的二元溶劑系進行菌絲纖維的溶解，發現此溶劑系中氯化鋰的濃度在5%左右時菌絲纖維的可達到約爲1.5%的一定值。之後，再以所得菌絲纖維的均相溶液進行成膜工程。成膜後可得20-150mm不同膜厚的菌絲纖維膜及殼糖/菌絲纖維膜，二種膜皆爲淺褐色。之後，以13C CP-MAS NMR進行菌絲纖維的結構解析，由所得的光譜可知其具有兩種可能性，（1）由部份的poly(N-acetyl-b-D-glucosamine)與部份的poly(b-D-glucosamine)共構成脫乙醯化度較高的共聚物(Copolymer)、（2）由b-1,3Glucan與poly(N-acetyl-b-D-glucosamine)所構成，而結構的再確認則在本計劃執行的第二年以不同製備方法所得的白色菌絲纖維膜進行結構的比對分析。在膜的物理性質方面，所得的試樣經拉力試驗後發現菌絲纖維膜、菌絲纖維/殼糖膜及非均相白色膜的強力分別爲900、1500、600 gf/mm2左右，可知由均相比非均相成型所得

的試樣強力爲高，並且在加入殼糖的均相膜也會因殼糖的高分子量提高菌絲纖維膜的強力。在表示親水性與膨潤性的動態接觸角測定後發現均相膜與加入殼糖的試樣，與水的動態接觸角與濕張力都維持在75度、20dyne/cm左右但白色膜的動態接觸角及吸濕的速度卻是十分地快速，這可能是由於白色膜是以非均相法製得，因此其具有低密度及大孔隙度所致。本子計劃的第二年將著重於菌絲纖維的結構鑑別、菌絲纖維的抽絲嘗試作爲吸收性手術用縫線的製備及相關性質的探討。

貳、八十八年度計畫著作一覽表

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於：□子計畫　　☑總計畫(請自行整合)

列出貴計畫於本年度中之所有計畫產出於下表，包含已發表或已被接受發表之文獻、已取得或被接受之專利、擬投稿之手稿（manuscript）以及專著等。「計畫產出名稱」欄位請依「臺灣醫誌」參考文獻方式撰寫；「產出型式」欄位則填寫該產出爲期刊、專利、手稿或專著等，舉例如下：

序號	計畫產出名稱	產出型式	SCI*	致謝與否
例	Chang SF, Cheng CL. The suppression effect of DNA sequences within the C4A region on the transcription activity of human cyp21. Endocrine research. 1998, 24(3&4):625-630	期刊	V	V
1.	Su CH, Sun CS, Juan SW, Ho HO, Hu CH, Sheu MT. Development of fungal mycelia as skin substitutes:effects on wound healing and fibroblast.Biomaterials 1999.20:61-68	期刊	V	
2.	Su CH, Sun CS, Juan SW, Ho HO, Hu CH, Sheu MT. Development of fungal mycelia as skin substitutesII:effects on wound healing.1999.1(1):40-46	期刊		
3.	Tseng H, Lu JL, Su CH. Evaluation of Chemical Properties of Membrane from Ganoderma. Proceding of The 4th Biomedical Materials & Technology Symposium 1997, p13	研討會		
4.	Lin SC, Su CH, Tseng H. Study on Preparation of Membrane from Fungal Ganoderma Residue as Skin Substitute and its Solubility and Mechanical Properties. 臺北醫學院 87 年度師生聯合學術研究發表會 1998, A30	研討會		
5.	Analysis of cytotoxicity of Sacchachitin and its effects on local immuno-modulation and inflammation during skin wourd healing	研討會		
6.				

*SCI：Science Citation Index，若發表之期刊爲 SCI 所包含者，請打勾。

*「致謝與否」欄位：若該成果產出有註明衛生署或國家衛生研究院委託資助字樣者，請打勾。

參、八十八年度計畫重要研究成果

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於：□子計畫　　☑總計畫(請自行整合)

1.計畫之新發現或新發明

每一種生醫材除具備良好的生理特性與活性,因此當這些生醫材被進行利用時,研究開發人員都希望能發揮其構思進行各種賦型。而賦型的最佳方法不外乎熔解與溶解。多醣由於其立體結構的不同及多數的分子間及分子內氫鍵, 使其結構安定性造成不熔解及不溶解性,因此每種多醣類難找到良好的溶劑系 ;當然,靈芝這種多醣亦不例外。過去從未有有關靈芝的文獻,本研究發現了靈芝的 LiCl/DMAC 的二元溶劑系,並且對此二元溶劑系進行各種性質的探討。其中並發現由均相溶液製備出的靈芝膜可以更薄、更強。

2.計畫對學術界或產業界具衝擊性（impact）之研究成果

由於靈芝的殘餘菌絲纖維是一種複合醣質,且其具特有的生理活性,因此本研究對於靈芝的二元溶劑系的開發得到初步性的成功。除了作爲本研究中的人工皮膚外,在作爲褥瘡治療藥物致效用等用途或進一步與各種蛋白質的結合成爲更高階的醫藥等,均相溶液卻提供了一個高反應率的方法。

肆、八十八年度計畫所培訓之研究人員

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於: ☐子計畫　　☑總計畫(請自行整合)

種類			人數	備註
專任人員	1. 博士後研究人員	訓練中	1	
		已結訓		
	2. 碩士級研究人員	訓練中		
		已結訓	2	
	3. 學士級研究人員	訓練中	2	
		已結訓	2	
	4. 其他	訓練中	2	臨時工
		已結訓		
兼任人員	1. 博士班研究生	訓練中		
		已結訓		
	2. 碩士班研究生	訓練中	1	
		已結訓		
醫師		訓練中		
		已結訓		
特殊訓練課程				

伍、八十八年度重要研究成果產出統計表

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於: □子計畫　　☑總計畫(請自行整合)

(係指執行八十八年度計畫之所有研究產出成果)

科技論文篇數			技術移轉			技術報告
	國內	國外	類型	經費	項數	篇
期刊論文	1 篇	篇	技術輸入	千元	項	技術創新 項
研討會論文	1 篇	篇	技術輸出	千元	項	著作權(核准) 項
專著	篇	篇	技術擴散	千元	項	專利權(核准) 項

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部分，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內。

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者。

專　　著：爲對某項學術進行專門性探討之純學術性作品。

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者。

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散。

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者。

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種。

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）。

陸、參與八十八年度計畫所有人力之職級分析

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於: □子計畫　　☑總計畫(請自行整合)

職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	人
第二級	副研究員、副教授、總醫師	4人
第三級	助理研究員、講師、住院醫師	2人
第四級	研究助理、助教、實習醫師	3人
第五級	技術人員	2人
第六級	支援人員	人
合計		11人

〔註〕

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者。

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Biomaterials

Development of fungal mycelia as skin substitutes: Effects on wound healing and fibroblast

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Abstract

In this study, Sacchachitin membrane, prepared from the residue of the fruiting body of *Ganoderma tsugae*, was estimated for its effects on wound healing and the proliferation and migration of fibroblast cells. Two mirror-image wounds were made on the back of female guinea pigs by dissecting a 1.5×1.5 cm^2 skin surface of full thickness. Sacchachitin membrane was placed randomly on one of the wounds and gauze or Beschitin® on the other. Changes in the wound area were measured and photographed after a predetermined amount of time postoperatively. Histological examination of the wound and surrounding tissue was also performed to reveal any interaction of tissue with the dressing. The results showed that the wound area covered with Sacchachitin membrane was statistically smaller than that covering with gauze on day 10, whereas there was no significant difference in the wound size compared to that with Beschitin®. Fibroblast cells from the dermis layer of guinea pigs were used. The number of fibroblast cells were counted on the predetermined days in the culture suspended with or without 0.01% w/v dressing materials. By layering on DMEM plates, the number of fibroblast cells migrating across the center line or outside of the central hole were counted after five days. All the results indicated that both 0.01% w/v of Sacchachitin and chitin significantly enhanced the proliferation and migration of fibroblast cells.
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Keywords: *Ganoderma tsugae*; Fungal mycelia; Wound healing; Fibroblast; Proliferation; Migration

1. Introduction

Wound healing is defined as the restoration of the continuity of living tissue and is an integrated response of several cell types to injury. It involves platelet aggregation and blood clotting, the formation of fibrin, an inflammatory response, alteration in the ground substance, endothelial and capillary proliferation and surface covering, regeneration of certain cell types, variable contracture and remodeling [1]. Healing is not complete until the disrupted surfaces are firmly knit by collagen. Generally, the use of a skin substitute to provide an environment conducive to healing is necessary [2]. In an endeavor to develop an ideal skin substitute, the performance of Sacchachitin membrane, prepared from the residue of the fruiting body of the medicinal fungus, *Ganoderma tsugae*, as an effective skin prosthesis has been examined [3]. This study evaluated the effectiveness of Sacchachitin membrane in the management of excised wounds in guinea pigs and compared its performance with gauze and Beschitin®.

A variety of wound models have been employed to study the wound healing process. The techniques that have been employed involve morphological examination of the wound size, histological examination of biopsied tissue samples, the detection of collagen content, the number of cells in the new connective tissue and epithelial layers [2], and the measurement of some biochemical parameters [1]. In conjunction with the area measuring technique and histological examination, evaluation of the effect of Sacchachitin on the proliferation and migration of fibroblasts in culture was included.

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2. Materials and methods

2.1. Materials

The residue of the fruiting body of *Ganoderma tsugae* was collected after hot water extraction twice and was a generous gift from a factory in Natuao, Taiwan. Beschitin-W®, made from crab chitin, is a commercial product of Morihita Resere Co. (Japan). Ketamine HCl and xylazine were supplied by Sigma Co. (St. Louis, MO, USA). Female guinea pigs, weighing from 380 to 480 g and aged from 8–10 weeks, were purchased from the Animal Center, National Taiwan University. Analytical-grade reagents were obtained from Merck Co. (Germany). Deacetylated Sacchachitin was obtained by dissolving Sacchachitin in 45% NaOH with heating. The resulting solution was dialyzed with tap water for two days and then with distilled water for one day resulting in a solution with a neutral pH. This solution was then freeze-dried to obtain deacetylated Sacchachitin. β-Glucan was the alkaline-soluble fraction of the polysaccharides obtained by treating the residue of the fruiting body of *Ganoderma tsugae* with 1 N NaOH at 90°C for 4 h.

2.2. Preparation of Sacchachitin membrane

The purification of fibers to form Sacchachitin membrane was followed a similar procedure that reported in the previous paper [3], except that the treatment with 1 N NaOH was conducted at 90°C for 4 h. The fibers with lengths in the range of 10–50 μm were then collected and dispersed in deionized water to form a suspension. The suspension was then filtered. The membrane formed on the filter paper was then freeze-dried (EYELA, model FD-5N) to obtain a porous membrane with a diameter of 7 cm and thickness of 0.1–0.2 mm for the following studies. The chemical constituents of the final product was determined to be 40% *N*-acetyl-D-glucosamine and 60% β-1,3-D-glucan. The membranes were autoclaved and kept under aseptic conditions until use.

2.3. Wound healing studies

Prior to the study, guinea pigs were anesthetized separately with Ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}) by abdominal injection. The dorsal and abdominal hairs of the guinea pigs were removed with an electric razor. The method proposed by Kaufman was followed to prepare wounds [4]. Two equal mirror-image areas were marked on the dorsal area of the guinea pigs 1 cm apart from the spinal cord and in between the 12th rib and iliosaacral joint. Two pieces of full thickness skin, each with a surface area of about 1.5 × 1.5 cm^2, were excised. The method of excision was similar to that reported by Smahel [5]. The depth of the excised area was as deep as the panniculus carnosus. After cleansing off the blood residues with gauze and 0.9% saline solution, one of the lesions was randomly chosen and covered with an equal size of cotton gauze or Beschitin for comparison. The other side was covered with Sacchachitin membrane as prepared above. Both dressings were hydrated with 0.9% saline solution to promote the adhesion of the dressings to the wound surface. Treated guinea pigs were placed in individual cages with an air-filtering device in a temperature range between 22–28°C with humidity control.

After surgery, the area of the wound was measured on the 5th, 10th, 15th, and 20th days. Fresh dressings were replaced at the same time. A modified method of Nangia to calculate the wound area was employed [2]. Generally, the outline of the wound area was marked on a transparent paper and then transcribed to another piece of ordinary paper. A hand-held scanner (Proscan Gray) was used to capture the image and data were stored as a monochromic BMP file. With proper adjustment, a computer program written with Visual Basic was employed to calculate the wound area so obtained. A total of 15 guinea pigs were included in this study. The statistical significance of any difference was analyzed by a paired Student's *t*-test.

2.4. Histological analysis

On days 5, 10, 15, and 20, one of the guinea pigs from the above study was killed. Lesions with the surrounding tissue were excised in a deep-V shape. Specimens were then fixed in 36% formalin for 2–3 h and then cut into two halves to promote the infiltration of formalin into the tissue. Specimens were dehydrated with a tissue auto-treatment device (Sakura, RH-12E) and embedded in paraffin with a Paraffin Dispenser (Shadon, Lipshow). Sections of appropriate thickness (about 5 μm) were sliced (Sakura, IVS-400) and stained with haematoxylin and eosin. Entellan (Merck) was used to seal the specimen before examination using a Hitachi model S-2400 SEM.

2.5. Implantation

The implantation was according to the modified method proposed by Peluso [6]. All animals were anaesthetized in the same way as described above. A 1 cm square of autoclave-sterilized Sacchachitin was implanted into the lesion between the subcutaneous tissue and muscular membrane on the dorsal area of the guinea pigs 1 cm away from the spinal cord and in between the 12th rib and iliosaacral joint. On the opposite side, either gauze or Beschitin was implanted as a control. Both lesions were then closed with 3-O nylon sutures. Guinea pigs were kept in individual cages and the sutures removed on day seven. On day 14, the implants were excised together with the surrounding tissue. The specimens

were then fixed in 36% formaldehyde for 2–3 h and embedded in paraffin. Sections of appropriate thickness were then sliced and examined using a Hitachi model S-2400 SEM.

2.6. Scanning electronic microscopy (SEM) examinations

The Sacchachitin membrane samples harvested on day 9 from lesions were washed three times for 15 min with 0.1% cacodylate buffer solution (pH 7.4) containing 7% sucrose. The samples were then fixed in cacodylate buffer solution containing 1% OsO_4 for 1.5 h. After that, the samples were washed again according to the same procedure as described above. The specimens were dehydrated by immersion in a series of aqueous solutions of increasing alcohol content, followed by critical point drying using liquid CO_2 as the transfer medium (Hitachi, HCP-2). Dried samples were then loaded on aluminum studs and coated with gold for 3 min at 8 mA under a pressure of 0.1 Torr (Hitachi, IB2). The samples were scanned and examined using a Hitachi model S-2400 SEM. One control sample was prepared by simply immersing another Sacchachitin membrane in phosphate buffer for nine days and a second control sample was untreated Sacchachitin.

Part of the Sacchachitin membrane obtained from the wound healing studies was treated with 10% NaOH to remove blood clots and was then cleaned with distilled water until the pH was neutral. Membranes were pressed between two glass slides and treated with 0.5% periodic acid for 5 min. After that, they were washed with water several times and then incubated in Schiff's solution for 15 min. After washing with water for 10 min, a purple color could be visualized on these membranes. A control sample of Sacchachitin stored in phosphate buffer solution was also prepared.

2.7. Preparation of fibroblast cells

Fibroblast cells were acquired by the method of primary culture. A piece of skin was isolated from the dorsal area of a guinea pig and sterilized in iodine–alcohol solution for 30 s and then in 70% alcohol for 15 s. It was then cut into several pieces each with a surface area of about 2 mm^2. After attaching to a tissue culture dish for 20 min, 10 ml of culture medium (DMEM with 15% v/v fetal bovine serum, 1% v/v streptomycin) was added followed by storage for a couple of days in an incubator (Sanyo, MC0175) controlled at 37°C and with 5% CO_2. Fibroblast cells were harvested simply by removal of skin specimens [7].

2.8. Proliferation studies of fibroblast cells

Fibroblast cells at a concentration of about 3×10^4 cells ml^{-1} was placed in the DMEM medium in a petridish 35 mm in diameter. After incubation for 24 h, the medium was changed with fresh medium containing various materials at a concentration of 0.01% w/v. The materials tested included Sacchachitin powder, deacetylated Sacchachitin, chitin powder, *N*-acetyl-D-glucosamine, and *β*-1,3-D-glucan. On a predetermined day, 0.25% Trypsin-EDTA was added to detach the cells. About 50 μl of cell suspension was sampled and mixed with an equal volume of 0.5% Trypan blue. The total number of live cells was then counted using a hemocytometer under light microscopy [8]. A growth curve was plotted for each material added to the medium. The results were an average of replicated samples. At the same time of sampling, 70% of the medium was replaced with fresh medium. On day 9, another cell suspension was sampled and fixed with 70% aqueous alcohol solution followed by the addition of 300 μl of propidium iodide solution (50 μg ml^{-1}). The number of cells in the solution was counted for 5 min by a flowcytometer (FACScan, Becton Dickson). Data was analyzed with LYSIS 2 software. The effects of two different concentrations (0.05 and 0.1% w/v) of *N*-acetyl-D-glucosamine, deacetylated Sacchachitin and *β*-1,3-D-glucan, were also examined.

2.9. Migration studies of fibroblast cells

Fibroblast cells (3×10^5 cells ml^{-1}) were incubated in the DMEM medium containing 0.2% FCS for 72 h in a petridish. Aphidicolin (0.5 mg ml^{-1}) was added and the incubation continued for another 24 h. About 1.2×10^5 of these cells were incubated in a petridish of 35 mm in diameter until the cells were grown fully over the whole dish area. A cell lifter was used to mark a cross line in the center of the dish and those cells on one side were scratched off. The remaining cells on the other side were cleaned twice with phosphate buffer solution. Then, a medium containing 0.01% w/v of various materials (chitin, deacetylated Sacchachitin, and *β*-glucan) was added. Incubation was done at 37°C with 5% CO_2. The extent of migration was estimated by counting the number of cells growing across the central line at six different points each within an area of 600×600 μm^2. Differences among different materials were analyzed based on the Student's *t*-test with $P < 0.05$ [8–10].

Another way of estimating the migration of fibroblast cells was as followed. The test materials suspended in 1% agarose medium were placed in a petridish and a layer consisting of a precipitate of the test material later formed at the bottom of the petridish. A hole was punched with a Pasteur pipette in the center of the solidified agarose medium. About 1000–1500 cells were placed into the hole, 15 min later, the cells precipitated to the bottom of the hole. The hole was then covered with DMEM medium and incubated at 37°C with 5% CO_2 for a certain period of time. From the center of the hole, the cells that migrated along the interface between the layers of

agarose and of the test material were counted in six different areas of equal size but randomly chosen [11–12]. The Student's *t*-test was used to analyze any significant difference among the materials tested.

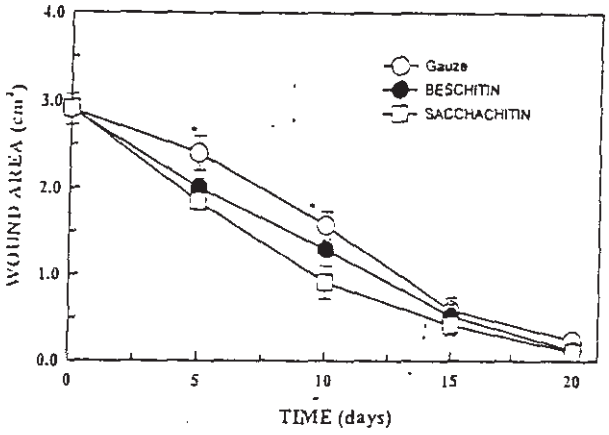

Fig. 1. Comparison of wound area changes at different time intervals when using Sacchachitin, Beschitin and gauze to cover the wound. *: a paired *t*-test of significant difference with $P < 0.05$).

3. Results and discussion

Changes in the wound area covered either with Sacchachitin, Beschitin or gauze were estimated on days 5, 10, 15, and 20. The average change in the wound area is shown in Fig. 1 and demonstrates that the wound area covered with Sacchachitin measured at the above mentioned time was smaller than that covered with gauze. However, the difference between the area covered with Sacchachitin and that with Beschitin was not significant. Histological examination of wound tissue showed that new cells were apparently formed on day 10 in wounds covered with both Sacchachitin or Beschitin. On day 20, the differentiation of the hair follicles was also observed. However, the new cells only appeared on day 15 in wounds covered with gauze with shrinking of the wound area being observed also. Histological examination further revealed that the infiltration of numerous polymorphonuclear leukocytes into the wound area covered with Sacchachitin and Beschitin, whereas only a few monocytes were found to have infiltrated the wound area covered with gauze. These results are shown in Figs. 2–4.

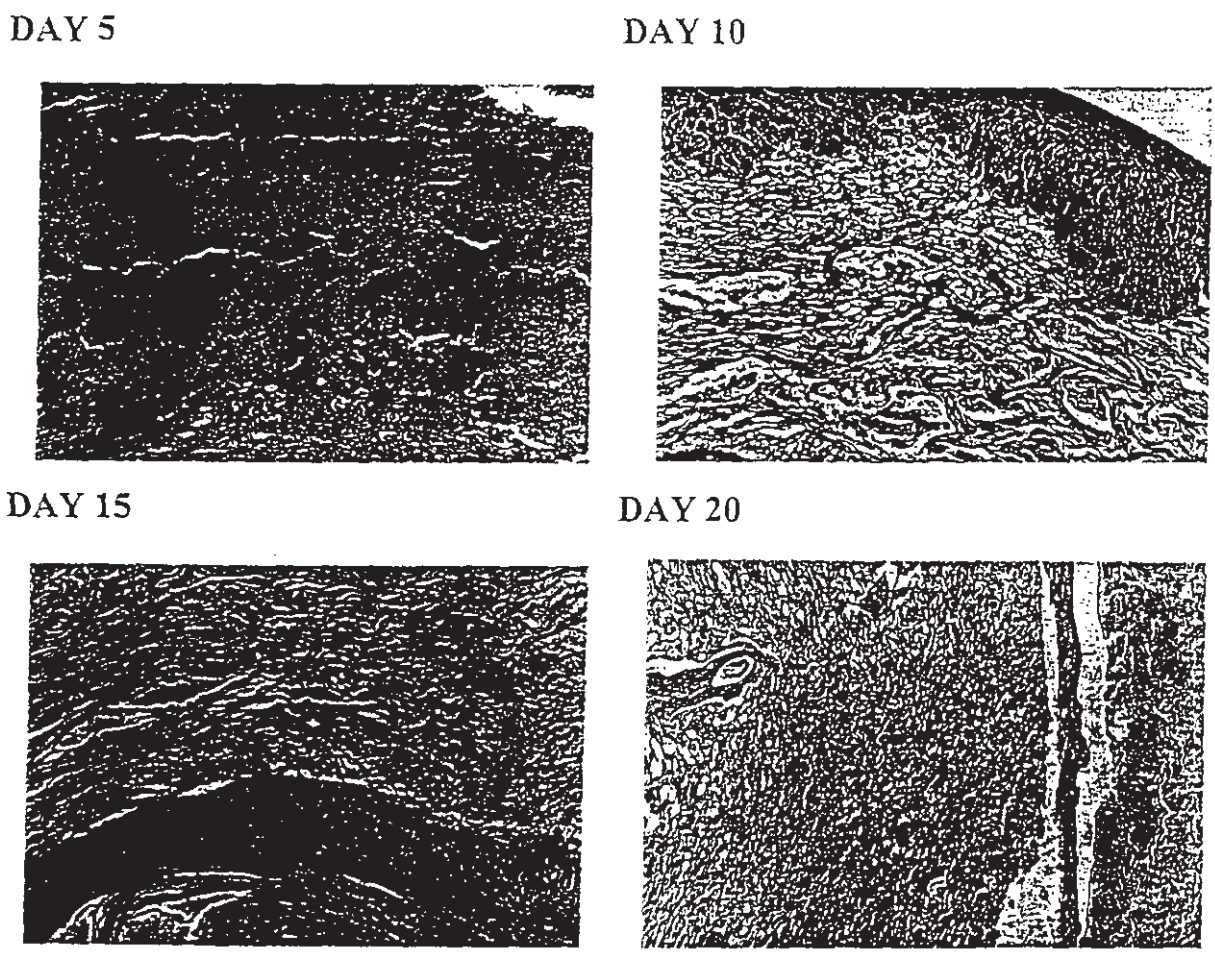

Fig. 2. Photomicrographs of the wound area and its surrounding tissue covered with Sacchachitin membrane at different time intervals.

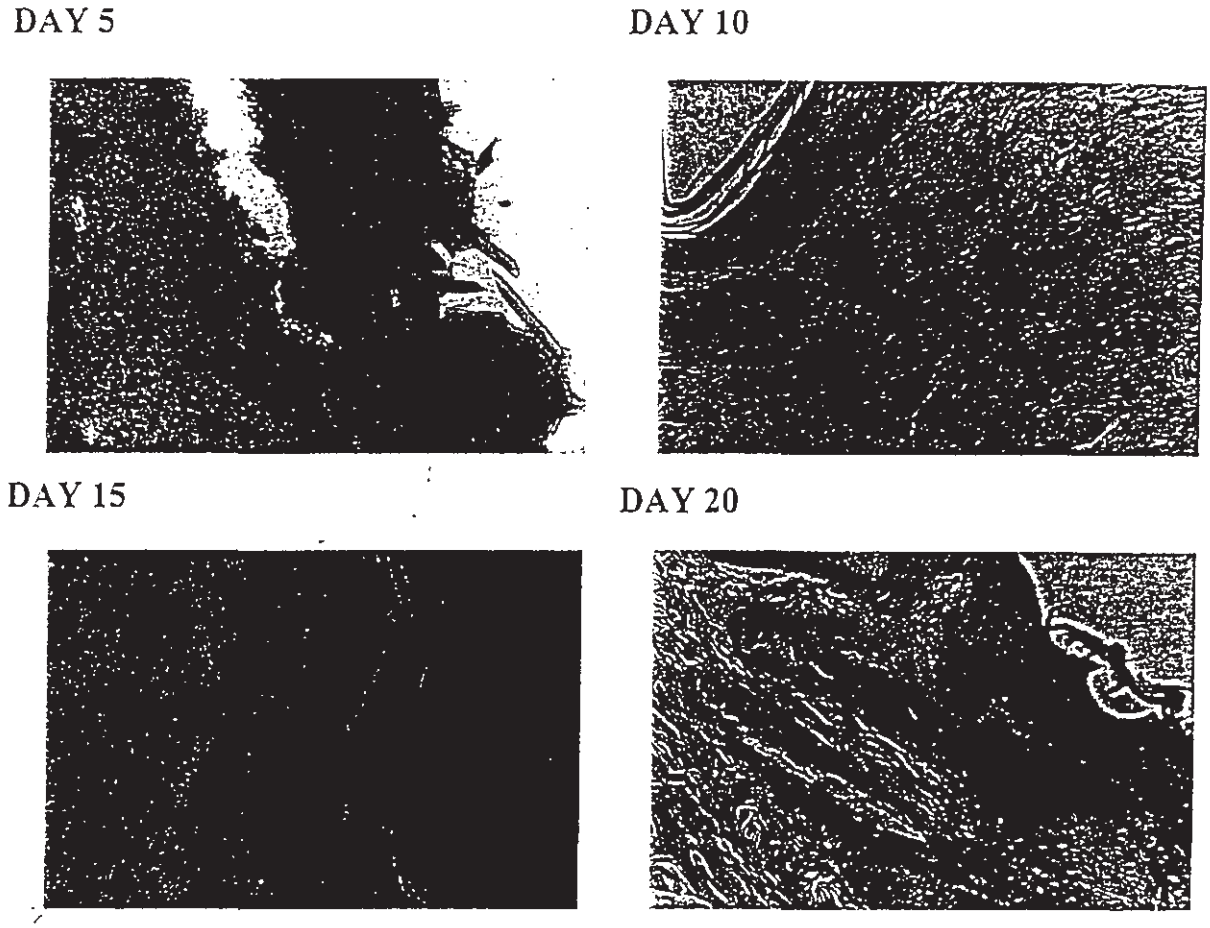

Fig. 3. Photomicrographs of the wound area and its surrounding tissue covered with Beschitin membrane at different time intervals.

The accumulation of polymorphonuclear leukocytes, such as neutrophils and eosinophils, in wound areas covered with either Sacchachitin or Beschitin is a sign of an acute inflammatory reaction. However, no bacterial infection was observed, indicating that both materials had a chemotactic effect on the inflammatory cells. On the contrary, the infiltration of a large amount of monocytes, i.e. lymphocytes and macrophages, into the wound area covered with gauze is attributed to a type IV allergic reaction. This is a type of rejection phenomenon involving the activation of these chronic inflammatory cells causing necrosis of the wound tissue or the appearance of blisters on the skin. In 1976, it was reported that a mild acute inflammatory reaction of a wound caused by infection in the earlier stage of trauma was able to accelerate healing of the wound and to increase the ability to tolerate the tension after healing as well [13]. It was also found that numerous polymorphonulcear leukocytes with some macrophages, which are able to secrete cell cytokines or growth factors, had accumulated in the vicinity of the wound. Possible reasons proposed for the acceleration of wound healing by the author was attributed to the action of cell cytokines or growth factors on promoting the differentiation of granuloma granulation tissue in the wound area [13]. This would explain the acceleration of wound healing by Sacchachitin membrane and Beschitin.

When Sacchachitin membrane isolated from the wound area was examined, it appeared that the structure of the mycelia hypha were destroyed and would not stain with PAS (Fig. 5A). On the other hand, the untreated Sacchachitin membranes and that immersed in phosphate buffer solution (Fig. 5B) showed no signs of damage to the structure of the mycelia hypha and they could be stained with PAS. Thus, it appears that the constituents of Sacchachitin membrane that can be stained by PAS were eliminated during the wound healing process.

It has been known that chitin is hydrolyzable by lysozyme [14]. Since chitin is a portion of the Sacchachitin structural unit with some soluble polysaccharides, it appears likely that substances released by the infiltration of body fluid caused dissolution of the chitin to promote wound healing. SEM examination confirmed that the structure of the mycelia hypha was disintegrated and would not stain with PAS. Generally, polysaccharides with 1,6-linkage can be stained by PAS. The

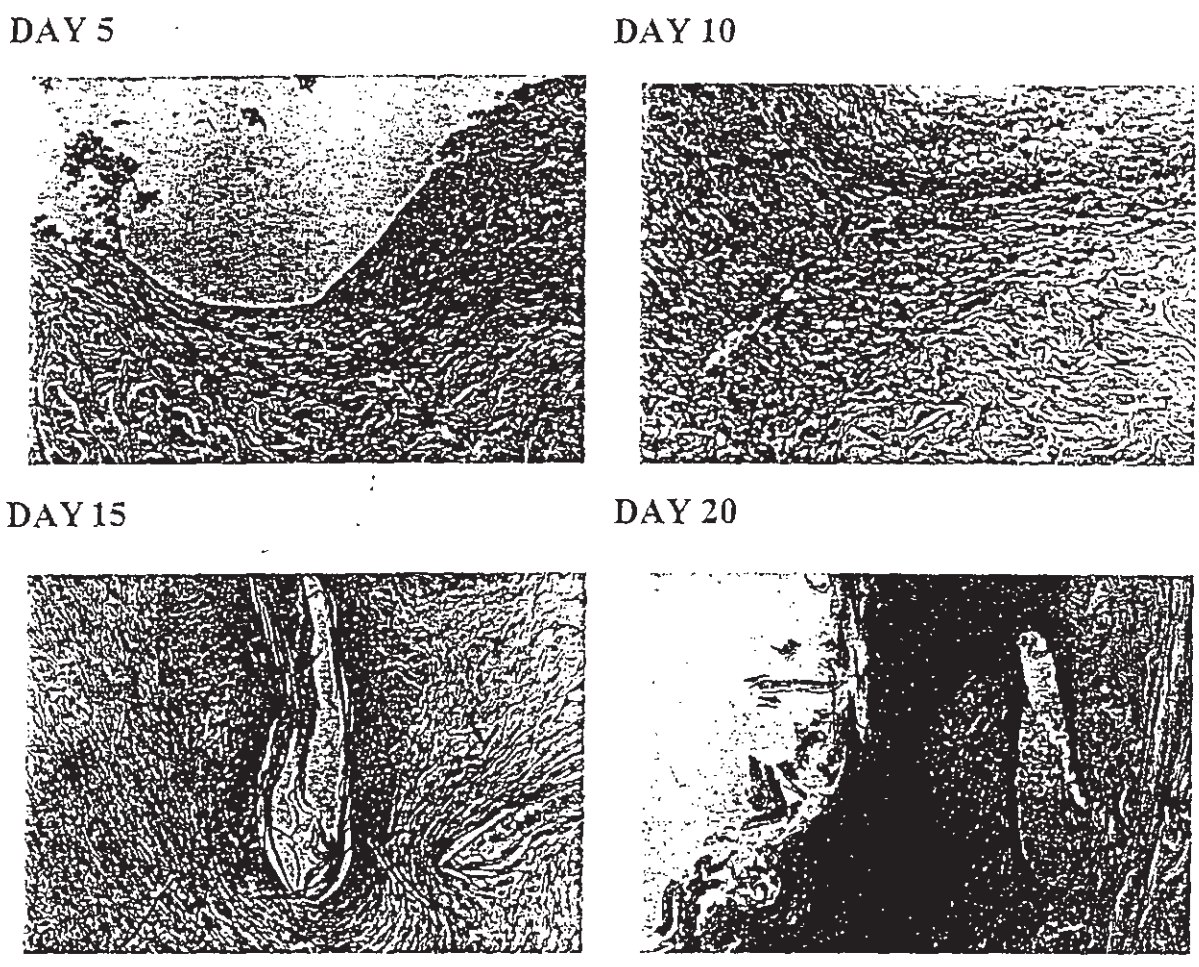

Fig. 4. Photomicrographs of the wound area and its surrounding tissue covered with gauze at different time intervals.

disappearance of the stainability of Sacchachitin membranes covering the wound indicates that this type of polysaccharide had disintegrated and been released. It is possible that the release of these substances can play an important role in the promotion of wound healing.

In order to determine which substances are responsible for the acceleration of wound healing, fibroblast cells, the main component of the dermis, were employed to compare the effect of several materials on their proliferation and migration. The results in Fig. 6 show that the proliferation of fibroblast cells was observed with both of 0.01% w/v Sacchachitin and Beschitin on day 6. Nevertheless, no significant effect on the proliferation of fibroblast cells was noticed until day 9 for 0.01% w/v *N*-acetyl-D-glucosamine and deacetylated Sacchachitin. It demonstrated that there is no difference for 0.01% w/v β-glucan compared to the control. The same results were obtained no matter which method was used for counting the number of cells.

The effect of different concentrations of these soluble materials on the proliferation of fibroblast cells was further examined and the results are plotted in Fig. 7. At a concentration of 0.05% w/v, *N*-acetyl-D-glucosamine and deacetylated Sacchachitin were able to promote the proliferation of fibroblast cells by day 6, whereas this did not occur until day nine for β-glucan (Fig. 7a). When the concentration of β-glucan was increased to 0.1% w/v, the proliferation of fibroblast cells occurred by day three, whereas the proliferation was inhibited with *N*-acetyl-D-glucosamine and deacetylated Sacchachitin (Fig. 7b).

In 1994, Chung et al. [15] reported that chitin isolated from the cell wall of some molds at a concentration of 0.01% w/v was shown to promote the proliferation of fibroblast cells. The same results were observed in this study. In 1987 [16] and 1992 [17], β-glucan was reported to be capable of promoting cell proliferation and macrophage function. However, the promotion of the proliferation of fibroblast cells was not observed until the concentration of β-glucan was increased above 0.05% w/v in this study. On the contrary, increasing the concentration of *N*-acetyl-D-glucosamine and deacetylated Sacchachitin to 0.1% w/v resulted in inhibition of fibroblast cell proliferation. In 1994, the same phenomenon was observed by Richard et al. [15]. Promotion of the

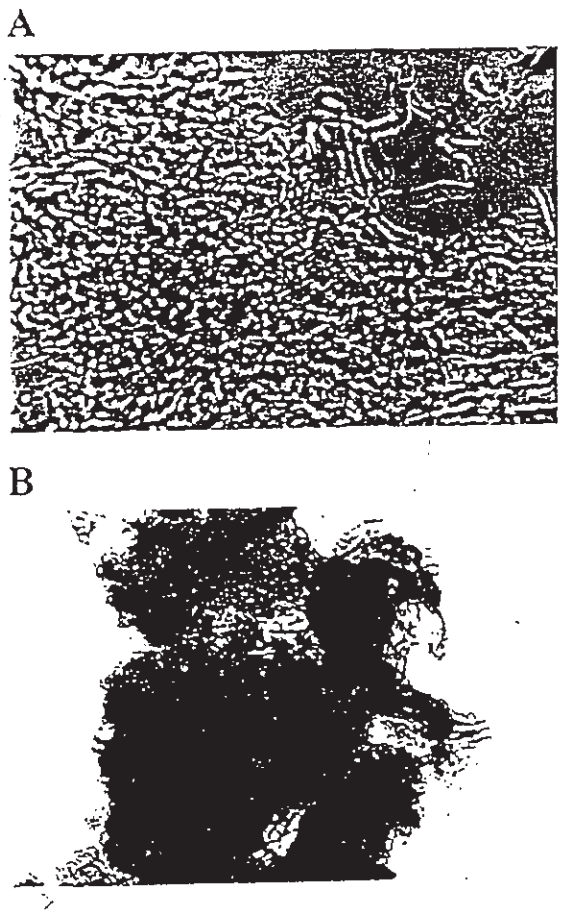

Fig. 5. Photomicrographs of Sacchachitin membranes covering the wound (A) immersed in phosphate buffer solution (B) stained by PAS.

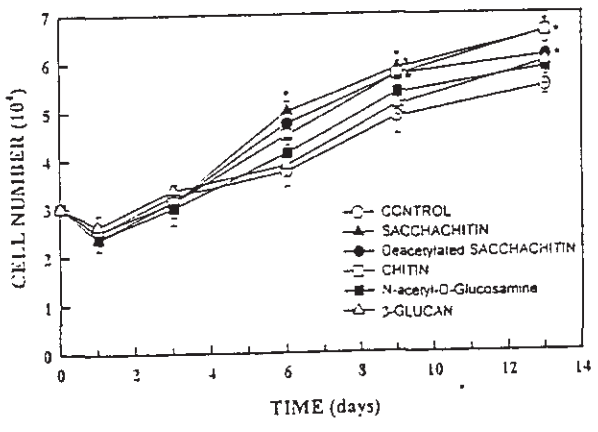

Fig. 6. Comparison of the proliferation of fibroblast cells induced by the addition of several different materials at a concentration of 0.01% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

proliferation of fibroblast cells has been attributed to the positive charge carried by chitin increasing the adhesion to the cell surface at lower concentrations. However, the interference of cell membrane function by the adhesion of

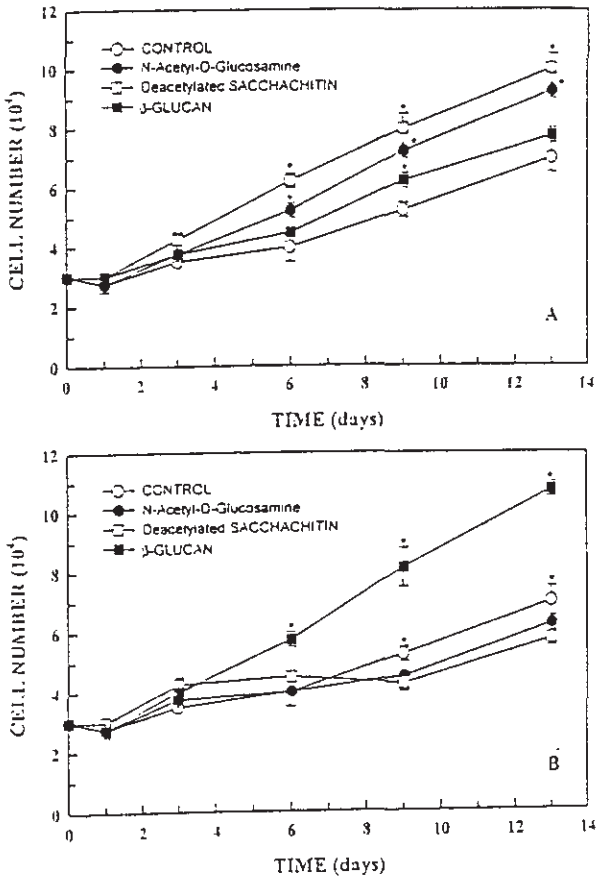

Fig. 7. Comparison of the proliferation of fibroblast cells induced by the addition of soluble materials at a concentration of (A) 0.05% or (B) 0.1% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

chitin leading to the death of cells was observed at higher concentrations.

Fibroblast cells controlled at the G_0/G_1 phase by aphidicolin were employed in two ways to estimate the extent of migration induced by different materials at a constant concentration of 0.01% w/v. In estimating the extent of migration across the central line in this study, it was found that Sacchachitin and chitin were able to promote extensive migration of fibroblast cells after the 3rd day. No difference in β-glucan from the control was noted. These results are shown in Fig. 8A. Similar results were observed in the migration study as estimated by counting the number of cells growing along the interface between the layers of agarose and the materials from the center hole in the medium (Fig. 8B). The only exception was that the cells migrating along the chitin layer were found to extract their peusdopodium and tended to die. Both results demonstrated that Sacchachitin membrane was able to promote the migration of fibroblast cells and act as a guide for cell growth.

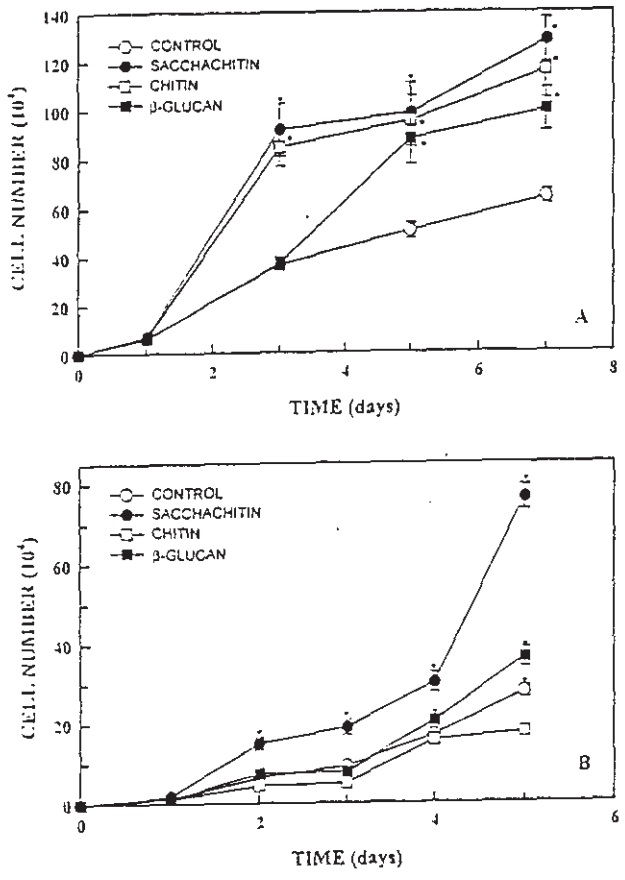

Fig. 8. Comparison of the extent of fibroblast cell migration (A) across the center line of the medium or (B) outside the central hole induced by the addition of soluble materials at a concentration of 0.1% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

4. Conclusions

Sacchachitin membrane is able to promote wound healing by inducing cell proliferation. A mild acute inflammatory reaction attracted a large number of polymorphonuclear leukocytes and some macrophages to clean away debris and blood clots. Also the secretion of cell cytokines and growth factors by these cells provided an excellent environment for wound healing. The migration of fibroblast cells, which was promoted by Sacchachitin, also plays another important role in the acceleration of wound healing. Optimally, the performance of Sacchachitin membrane as a skin substitute is comparable to the commercial product Beschitin. Further studies on the physical characteristics of the membrane, such as its air permeability and mechanical strength, would be valuable.

Acknowledgements

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Development of Fungal Mycelia as Skin Substitutes II: Effects on the Wound Healing Process

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Key Words
Ganoderma tsugae
Fungal mycelia
Wound healing
Macrophage
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ABSTRACT

The effect of SACCHACHITIN membrane, a skin substitute prepared from the residue of the fruiting body of *Ganoderma tsugae*, on wound healing was evaluated in this study. Two mirror image wound areas were excised on the back of female Wistar rats by dissecting a 2.0 × 2.0 cm^2 skin area of full thickness. SACCHACHITIN membrane was placed randomly on one of the wounds and gauze on the other. Changes in the wound area were examined after a predetermined amount of time postoperatively. Histological examination of the wound and surrounding tissue was also performed but only on the 4^{th}, 7^{th}, and 16^{th} days post-operation. The interaction of tissue with the dressing was evaluated by the implantation of these 2 materials. The results show that the wound area covered with SACCHACHITIN membrane was statistically significantly smaller than that covered with gauze for all time points measured. Histological examination revealed that SACCHACHITIN membrane induced mild inflammation and stimulated aggregation of polymorphonuclear leukocytes around the margin of the wound. The large number of macrophages and giant cells which infiltrated into the wound area covered with gauze indicates a response to a foreign body.

INTRODUCTION

One of the most urgent goals in the treatment of skin trauma is to provide an effective way of protecting any surface exposure of the skin. By covering with a suitable wound dressing, loss of body fluids is minimized, body temperature is maintained, infection of the wound area is controlled, and pain is relieved. Optimally, when the wound-healing process is accelerated, the original appearance and functions of the skin can be restored. Nowadays, the major method of treatment is skin grafting.However, the source of the

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skin and the cost of the medical treatment are problems that still need to be resolved. Substitution of skin with different materials as dressings appears to be an alternative choice.[1] Chitin (polymeric N-acetyl-D-glucosamine) is well known for its wound healing characteristics and has become one of the most important wound dressings in recent years.[2-5] Dating back to 1970, Prudden *et al.* demonstrated by their standard technical assay that chitin possesses an excellent acceleratory capacity for topical use, which was definitely superior to that of cartilage.[6] As a result, a whole new era was opened up for studying the wound-healing ability of chitin isolated from crab shell.[7-10] A product made of chitin from crab shell under the trade name BESCHITIN® W has been marketed by the Morihita Resere Co. of Japan.

Ganoderma tsuage, whose Chinese name is *Ling-zhi*, has long been an important member of the medicinal fungi used in the Asian area, including Taiwan and Japan.[11-13] However, after hot water extraction of the water-soluble fraction of *Ganoderma*, the resulting water-insoluble part (more than 90%) remains unused and is treated as waste. Recently, the mycelia components of Ganoderma were analyzed and revealed to be 40% chitin with 60% β-1,3-D-glucan.[14] Therefore, the possible functions of β-1,3-D-glucan and its synergetic effects with chitin could possibly make it an ideal biomaterial for use in wound dressings. In addition, since the extracted waste from *Ganoderma* contains the fibril structure of mycelia, it could be directly knitted into a membrane without requiring dissolving and fibril separation processes. The potential usefulness of this biomaterial as a wound dressing and its inherent advantages encouraged us to investigate its possible effects on the wound-healing process.

MATERIALS AND METHODS

Materials

The residue of the fruiting body of *Ganoderma tsugae*, a generous gift from a factory in Natuao, Taiwan, was collected after hot water extraction twice. Ketamine HCl was supplied by Sigma Co. (St. Louis, MO). Pentobarbital sodium was purchased from Siegfried Zofingen (Switzerland). Female Wistar rats, weighing from 300 to 410 g, were obtained from the Animal Center, National Taiwan Univ. Analytical-grade reagents were obtained from Merck Co. (Germany).

Preparation of the SACCHACHITIN Membrane

The purification of fibers to form the SACCHACHITIN membrane followed a similar procedure to that reported in a previous paper.[14] The fibers, with lengths ranging from 10 to 50 μm, were collected and dispersed in deionized water to form a suspension, which was subsequently filtered. The membrane formed on the filter paper was then freeze-dried (EYELA, model FD-5N) to obtain a porous membrane with a diameter of 7 cm and thickness of 0.1 to 0.2 mm for the following studies. The chemical constituents of the final product were determined to be 40% N-acetyl-D-glucosamine and 60% β-1,3-D-glucan. The membranes were autoclaved and kept under aseptic conditions until use.

Wound-Healing Studies

Prior to the study, rats were anesthetized separately with ketamine (35mg/kg) and pentobarbital (12 mg/kg) dissolved in water for injection via the abdominal route. The dorsal and abdominal hair of the rats was removed with an electric razor. The method proposed by Kaufman was followed to prepare skin trauma.[15] Two equal mirror-image areas were marked in between the 12th rib and iliosacral joint on the dorsal area of the rats and 1 cm from the spinal cord. Two pieces of full-thickness skin, each with a surface area of about 2.0 × 2.0 cm^2, were excised. The method of excision was similar to that reported by Smahel *et al.*[16] The depth of the excised area was as deep as the panniculus carnosus. After cleaning off the blood residues with gauze and 0.9% saline solution, one of the lesions was randomly chosen and covered with cotton gauze for comparison. The other side was covered with a SACCHACHITIN membrane as prepared above, being equal in size to the cotton gauze. Both dressings were hydrated with 0.9% saline solution to promote adhesion of the dressings to the wound surface. Treated rats were placed in individual cages with an air-filtering device in a temperature range between 22 and 28 °C with no humidity control.

After surgery, changes in the area of the wounds were measured on the 4th, 8th, 12th, 16th and 20th days,

after which fresh dressings were applied. A modified method of Nangia[17] to calculate the wound area was employed. Generally, the outline of the wound area was marked on a transparent piece of paper and then transcribed to another piece of ordinary paper.A hand-held scanner (Proscan Gray) was used to capture the image, and data were stored as a monochromic BMP file. With proper adjustment, a computer program written with Visual Basic was employed to calculate the wound area so obtained. A total of 15 rats was included in this study, and results were reported as means with standard deviation. The statistical significance of any differences was analyzed by paired Student's *t*-test. Another 8 rats were treated by the same procedure as that above except that the wound area was not measured until the 12th day. The results were also examined by paired Student's *t*-test. This served as a basis for examining the effects of mechanical injury on changes in the wound areas due to dressing replacement.

Histological Analysis

On days 4, 7, and 16, one of the 15 rats from the above study was sacrificed with pentobarbital (100 mg/kg). Lesions with the surrounding tissue were excised in a deep-V manner. Specimens were then fixed in 10% formalin for 24 h and embedded in paraffin. Sections of appropriate thickness were sliced and examined using a Hitachi model S-2400 SEM.

Implantation

Implantation proceeded according to a modified method of Peluso.[18] All animals were anesthetized in the same way as described above for the relief of any suffering due to pain during operation. Autoclave-sterilized SACCHACHITIN membranes and gauze were separately implanted into the dorsal area of rats on 2 opposite sides of the spinal cord (in between the 12th rib and iliosacral joint, with a depth reaching the panniculus carnosus). The lesions were then sealed with 3-O nylon sutures. Rats were kept in individual cages and sutures were removed on day 7. On day 14, the implants were excised together with the surrounding tissue. Specimens were fixed in 10% formaldehyde for 24 h and embedded in paraffin.Sections of appropriate thickness were then sliced and examined by both light microscopy and scanning electronic microscopy (Hitachi model S-2400 SEM).

Scanning Electronic Microscopy (SEM) Examinations

Specimens were dried by immersion in a series of aqueous solutions of increasing alcohol content, followed by critical point drying using liquid CO_2 as the transfer medium. Dried samples were then loaded onto aluminum studs and coated with gold for 3 min at 8 mA under a pressure of 0.1 torr. The samples were scanned and examined using a Hitachi model S-2400 SEM.

RESULTS

Changes in the wound area covered with SACCHACHITIN or gauze were estimated on days 4, 8, 12, 16, and 20. The results were plotted as shown in Fig. 1 and demonstrate that the areas of wounds covered with SACCHACHITIN membrane measured at the above time points were significantly ($p < 0.05$) smaller than those of wounds covered with gauze. Effects of mechanical injury on the change in wound area due to the replacement of dressings were examined and the results are shown in Fig. 2. Comparison of the wound area measured on day 12 with the corre-

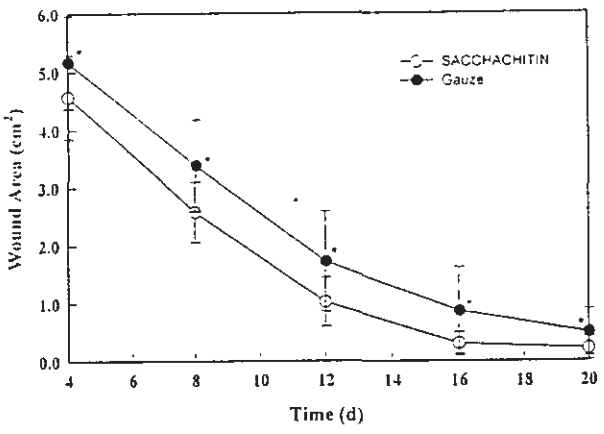

Fig. 1. Comparison of wound area changes between that covered with SACCHACHITIN membrane and with gauze at different time intervals. (*one-side paired *t*-test for significant difference with $p < 0.05$).

sponding data demonstrated no statistically significant effect of mechanical injury on the change in wound area ($p > 0.05$). This clearly indicates that any change in the wound area was not affected by mechanical stress imposed during the study, and that the data solely represents the difference between covering with SACCHACHITIN membrane and with gauze. Wound healing was thus accelerated when SACCHACHITIN membrane was used to cover the wound compared to when gauze was used.

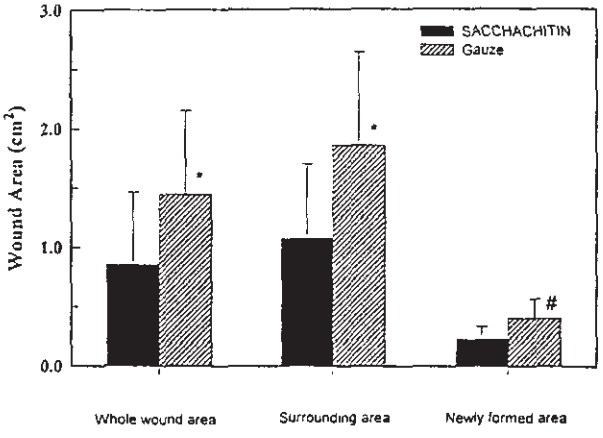

Fig. 2. Comparisons of the change of whole wound area, the area of newly-formed epithelia, and the surrounding area between that covered with SACCHACHITIN membrane and that with gauze on the 12th day. (*one-side paired t-test for significant difference with $p < 0.05$; # two-side paired t-test for insignificant difference with $p = 0.05$)

Histological examination of the wound tissue was conducted on days 4, 7, and 16. On the 4th day (Fig. 3a), a layer of exudate composed of polymorphonuclear cells and fibrous protein was found to have accumulated beneath the SACCHACHITIN membrane covering. Many new blood vessels had formed in the area close to the surface of the lesion, as well as underneath the newly growing epithelia. Inside the larger blood vessel, red blood cell stasis and margination of white blood cells were observed. A significant number of macrophages were present around the wound area. These phenomena indicate an acute inflammatory reaction and formation of granuloma granulation tissue. Fig. 3b shows similar phenomena for the wound area covered with gauze, except that a smaller amount of macrophages was observed.

By day 7, the growth of granuloma granulation tissue had become more apparent, and the presence of polymorphonuclear cells was observed in wounds covered with SACCHACHITIN membrane. Cell densities were higher and the matrix materials in the extracellular region had decreased. A photomicrograph is shown in Fig. 4a. In Fig. 4b, the existence of granuloma granulation tissue can be observed but not very clearly. In addition, the cell density was correspondingly lower in the wound covered with gauze compared to that covered with SACCHACHITIN membrane.

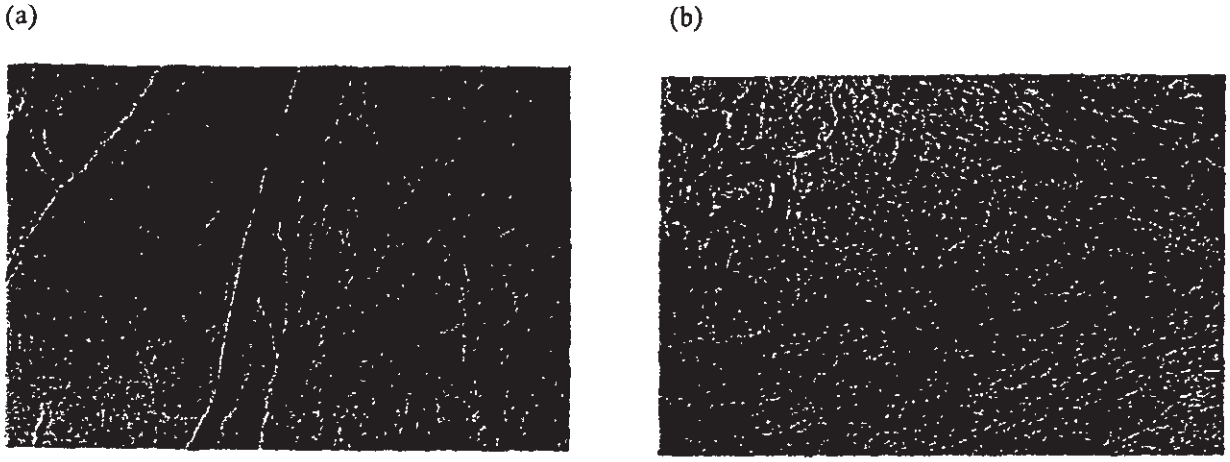

Fig. 3. Photomicrographs (× 100) of the wound area and its surrounding tissue covered with either SACCHACHITIN membrane (a) or gauze (b) for 4 days.

(a) (b)

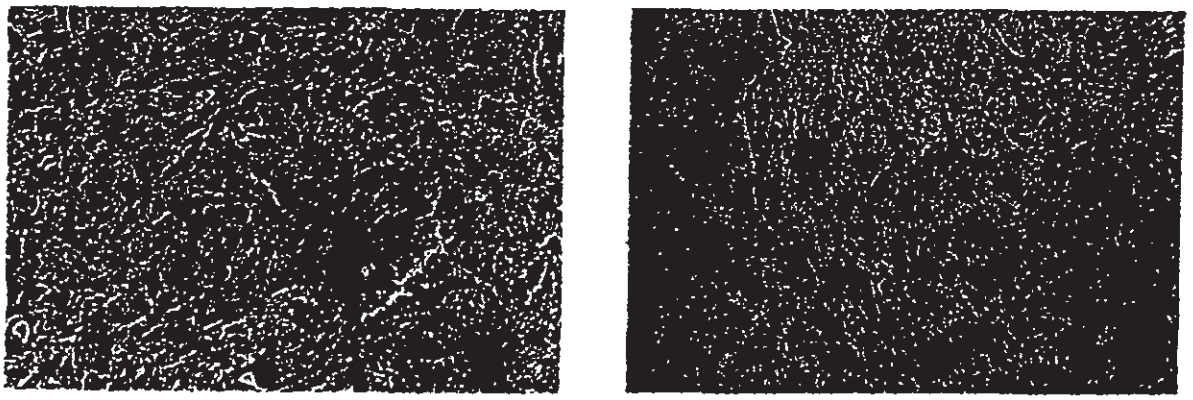

Fig. 4. Photomicrographs (× 100) of the wound area and its surrounding tissue covered with either SACCHACHITIN membrane (a) or gauze (b) for 7 days.

Figs. 5a and 5b show the results of the wound-healing process on day 16 when covered with SACCHACHITIN membrane for regions closer to and away from the center of the wound, respectively. The differentiation of the epithelia appeared to be quite good. A gradual change in the dermal region of the wound area was also observable: the closer the region to the center of the wound, the less the extent of differentiation of the epithelia observed, and granuloma granulation tissue appeared to be more obvious. Even then, the presence of mast cells fused with polymorphonuclear cells was observed; and in regions more distant from the center of the wound, the epithelia were well differentiated and a fibrous structure had formed with an abundance of matrix materials present extracellularly. On the contrary, granuloma granulation tissue was hardly observable on day 16 when the wound was covered with gauze (Fig. 6a). Only a small amount was found in the epithelia of regions closer to the wound. The dermal region had no mast cells and its major components were fibrous cells and extracellular matrix (Fig. 6b). However, the amount of extracellular matrix and level of cell density appeared to be lower compared to those observed with

(a) (b)

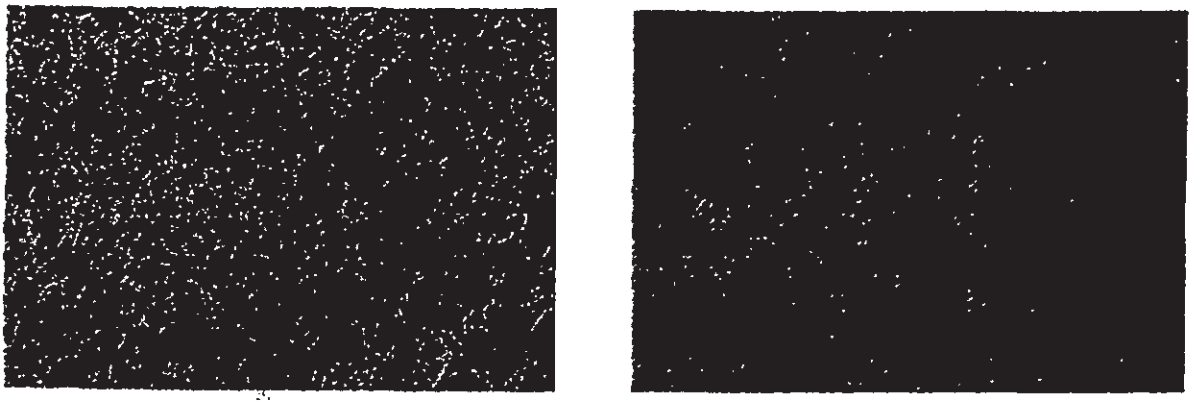

Fig. 5. Photomicrographs (× 100) of the region close to (a) or away from (b) the center of the wound area covered with SACCHACHITIN membrane for 16 days.

SACCHACHITIN membrane.

DISCUSSION

In 1977, Raju *et al.* suggested that an intense inflammatory response as a result of wound infection may greatly promote the differentiation of granuloma granulation tissue leading to better wound healing.[19] Franz *et al.* also discovered that a fetus wound infected by bacteria caused the same healing response as that observed in adults with the formation of a scar.[20] Although excessive inflammation leads to the death of tissue and a delay in wound healing, it is still recognized as beneficial to the body itself. It has been demonstrated that an appropriate inflammatory response can expel tissue debris, clean away foreign substances, minimize the chance of secondary infection, and promote the repair of tissue.[21-24] In the implantation study, an acute inflammatory reaction in the tissue was observed when the lesion was implanted with SACCHACHITIN membrane (Fig. 7a). It is believed that SACCHACHITIN membrane induces a similar phenomenon as a minor infection in the wound. This complies with what has been observed with the effect of SACCHACHITIN membrane on wound healing: on day 4, histological examinations revealed many more macrophages in the wound tissue. This was even more apparent on days 7 and 16. Furthermore, polymorphonuclear cells fused with macrophages were also apparent. This phenomenon of an accelerated wound-healing process is similar to that observed with the use of 20% benzoyl peroxide suspension[25] and also to that reported for chitin in the literature.[3]

In 1960, Prudden *et al.* discovered that cartilage was able to accelerate wound healing.[6] Furthermore, N-acetyl-D-glucosamine was shown by the same group to be responsible for acceleration of wound healing by chitin. Since SACCHACHITIN membrane is composed of 40% chitin and 60% β-1,3-D-glucan and a similar effect was observed in the wound healing process as with by chitin, its main mechanism of accelerating wound healing most likely can be attributed to its chitin component. Nevertheless, β-1,3-D-glucan itself is a strong activator of macrophages and is capable of attracting polymorphonuclear white cells.Since activated macrophages are known to accelerate wound healing,[26] the important role played by β-1,3-D-glucan can not be excluded.

Compared to SACCHACHITIN membrane, the macrophage reaction to foreign substances was the main response in the implantation study with gauze. Nevertheless, a smaller amount of macrophages was observed in a histological examination of the wound tissue covered with gauze. The main reason for this is still unclear. A possible reason is that a layer of thick crust, which was formed from the exudate secreted by the wound during an earlier stage, effectively prevented contact of the gauze with the wound.During the healing of the wound, the crust together with a major part of the gauze detached resulting in them not being recognized as a foreign substance (Fig. 7b).

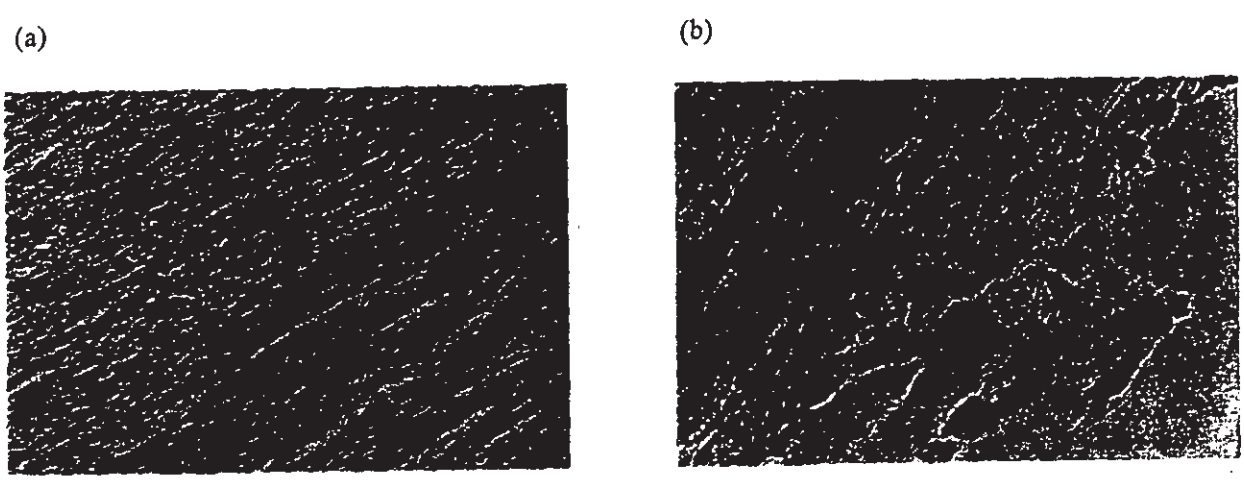

Fig. 6. Photomicrographs (× 100) of the region close to (a) or away from (b) the center of the wound area covered with gauze for 16 days.

A larger amount of exudate was found accompanying the wound when it was covered with SACCHACHITIN membrane. Furthermore, since chitin is able to attract an accumulation of polymorphonuclear white cells, a blue layer composed of cells involved in acute inflammation was found underneath the SACCHACHITIN dressing on day 4 during the implantation study. It appears likely that chitinase released by dead, active, or disintegrated granular white cells might be responsible for the disintegration of the SACCHACHITIN membrane. The debris from this disintegration was possibly eliminated mainly by macrophages. Along with drying of the wound, a solidified crust gradually formed and blocked the SACCHACHITIN membrane at the edges. Therefore, the main influence of the SACCHACHITIN membrane on the wound might be during the first few days. Furthermore, the disintegration of the SACCHACHITIN membrane by granulocytes might be a potential reason for the acceleration of the wound-healing process.[27]

Except for controlling infection and eliminating dead tissue, granulocytes have no obvious influence on the wound-healing process.[28] In a study on comparing the effect of GM-CSF and G-CSF on wound healing, GM-CSF simultaneously increased the number of both granulocytes and macrophages resulting in an acceleration of the wound-healing rate. Although G-CSF increases the number of granulocytes, it has no effect on the number of macrophages or the wound-healing rate.[29] Furthermore, for those patients with a low white cell count, the tolerance of wounds to tension during the healing process was found to be maintained normally. On the contrary, it has been recognized that macrophages play a more important role in the wound-healing process.[21,27,30,31] Not only are they involved in phagocytosis of foreign substances and the expression of antibodies, but secretion of cytokines by macrophages can also influence several different kinds of cell function. In the implantation study, an abundance of macrophages was found in the wound area covered with the SACCHACHITIN membrane. It would thus be reasonable to assume that the healing effect of SACCHACHITIN membrane on the wound was due to the activation of macrophages. The literature provides further evidence that chitin is able to activate as well as to enhance the function of ma-crophages.[18,32] In addition, it has been shown that chitin influences the epithelial cells of blood vessels, lymphocytes, fibrous cells, and the secretion of cytokines. Since the SACCHACHITIN membrane is similar in terms of chemical structure to glycosaminolycans, it may serve in guiding the reconstruction of the connective tissue.[33] All in all, the accelerating effects of SACCHACHITIN membrane on the wound-healing process can be attributed to an integrated response of all these functions.

CONCLUSIONS

In conclusion, a minor inflammatory reaction to-

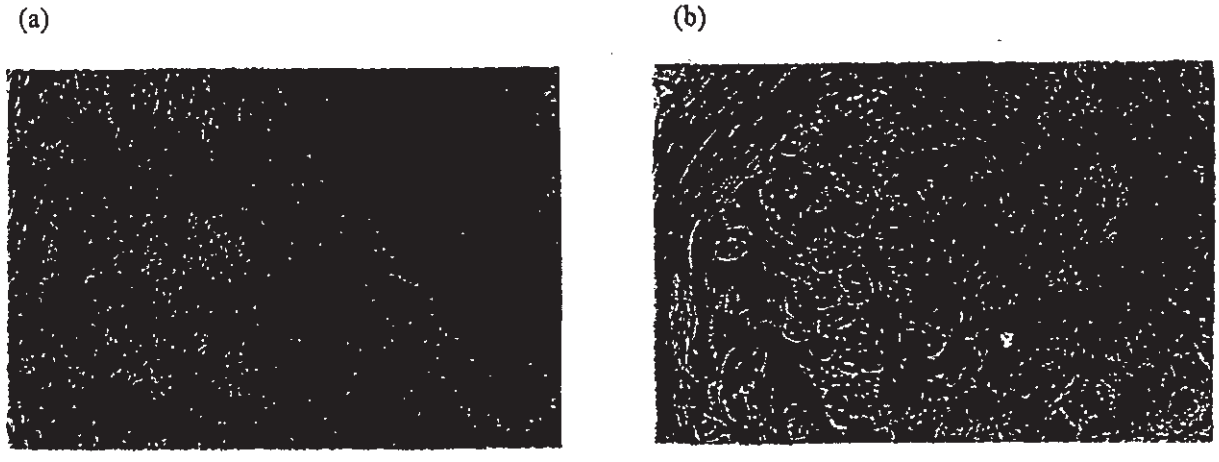

Fig. 7. Photomicrographs (× 100) of an acute inflammation response of the wound area to SACCHACHITIN membrane (a) and a foreign substance reaction of the wound area to gauze (b).

gether with the activation of macrophages to enhance the differentiation of granuloma granulation tissue are the possible mechanisms responsible for the acceleration of wound healing in the initial stage and for the increased tolerance in the wound area covered with SACCHACHITIN membrane. In several situations, such as chronic ulceration due to DM or bedsores, it would be preferable to have such a minor inflammatory reaction. On the contrary, excessive inflammation may cause the formation of scar tissue leading to a bad influence on the esthetic appearance of the skin. Fortunately, SACCHACHITIN membrane is just composed principally of fungal mycelia with no such differentiation ability as with bacteria.The self-limitating nature of SACCHACHITIN is another characteristic preventing excessive inflammation since it does not disintegrate markedly during healing of the wound.

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Evaluation of Chemical Properties of Membrane from *Ganoderma*

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Ganoderma, an important member of medicinal fungi, has recently been extensively studied for its biological activity and commercially marketed as healthy food. Usually, after removal of the water soluble fraction by hot-water extraction, the remaining water-insoluble part, mycelia fibers, (more than 90%) of *Ganoderma* was discarded.

The structure of mycelia fibers is relatively complicated comparing with most polysaccharides. Mycelia fibers are composed of b-1,3-glucan and poly (N-acetyl-b-D-glucosamine). Both b-1, 3-Glucan and poly (N-acetyl-b-D-glucosamine) can be absorbed and metabolized in our body. Pudden had demonstrated that poly (N-acetyl-b-D-glucosamine) has a beneficial effect in the wound healing. Moreover, oligo (N-acetyl-b-D-glucosamine), the enzymatic hydrolysis intermediate from poly(N-acetyl-b-D-glucosamine), has been shown to have a high adjuvant capacity. Su et al. have previously demonstrated that the skin dressing prepared from mycelia fibers, exerts a strong enhancement effect on wound healing. The properties of biodegradation, attractive biomedical material for medical uses than other synthetic materials. Taken together, these findings suggest that mycelia fibers might be used as the biomedical material for making skin substitute. However, there is not any solvent system suitable to dissolve mycelia fibers so far for the purpose of processing.

Since the chemical property of mycelia fibers is most partially similar to chitin, the same knowledge from our understanding in sacchachitin can be attempted to find the solvent system suitable for processing mycelia fibers. Most of the previously found solvent systems for chitin would induce molecular degradation except lithium salts/amino-containing solvents and calcuim salts/alcohol. In this investigation, we will apply the technique of casting to develop a facile type of sacchachitin membrane from the sacchachitin dope, which will be prepared with mycelia fibers dissolved in metal salt/amine-containing solvent. We believe that the success of this study will provide a very valuable new biomedical material for clinical use.

Key words: Ganoderma, sacchachitin, membrane, biomedical material, binary solvent system

以靈芝子實體殘渣作為人工皮膚取代物的薄膜製作、溶解機制及膜的機械性質探討

Study on Preparation of Membrane from Fungal Ganoderma Residue as Skin Substitute and its Solubility and Mechanical Properties

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靈芝長久以來為藥用真菌之重要成員，近年來更為生理活性之研究對象，並且於市面上以健康食品的方式廣泛販售。然而，在熱水萃取過程中取得水可溶部份之後，其餘至少 90%不可溶部份並無任何用途，成為廢料而被丟棄，由之前研究初步結果顯示，靈芝子實體纖維為主體的廢棄物可加工加以純化，並製成人工薄膜，而經由初步動物實驗證明其對大白鼠的皮膚切除傷口有促進迅速癒合的效果。其主要機轉可能來至靈芝子實體纖維所含的幾丁質及 1,3-β-glucan 複合體所引發的，此符合 1970 年 Pruden 發現 poly-N-acetyl-glucosamine(chitin)為創傷癒合促進的物質，也呼應日本利用螃蟹殼幾丁質作為人工皮膚創傷被覆材之商品例證。

本研究在探討溶劑中金屬鹽類對靈芝子實體纖維溶解行為的影響，進而對靈芝子實體纖維的萃取作最有效的利用。另一方面，利用萃取後的靈芝子實體溶液經過濃縮或和 chitin 溶液混合製成薄膜，除了提供動物試驗所需材料外，也利用 Dynamic Mechanical Analyzer（DMA,動態黏性分析儀）測定膜的機械性質。由所得結果顯示，溶劑中金屬鹽類的濃度及溶解過程中施予昇降溫的步驟可以明顯的增加靈芝子實體的溶解力。其中 5%LiCl/DMAc 溶劑可使靈芝子實體溶解力達到最高，並在溶解的過程中，經由昇降溫四次之後溶解力也有顯著的增加。由 DMA 靜力掃描（static force scan）測定結果中發現純靈芝膜（22.59 MPa）和以 1：1 體積比例加入 chitin 的靈芝膜（23.08 MPa）的斷裂點的單位施力則相差不大。此外，本研究亦利用 NMR 分析以利了解靈芝子實體之結構。

關鍵字：靈芝子實體、溶解力、人工皮膚、動態黏性分析儀

拾、附錄

子計畫-1（蘇慶華）

壹、實驗目的

大範圍皮膚缺損的處理一直是醫學界急於克服的目標。給予合適的覆蓋物可以減少體液的揮發、體熱的散失、傷口感染的機會以及解除病人的疼痛感；並期望能加速傷口的癒合，回復原先皮膚的美觀和功能。

靈芝長久以來爲藥用真菌的重要成員，近年來更成爲生理活性之研究對象，並且於市面上以健康食品之方式販售。然而，在以熱水萃取過程中取得水可溶性部份後，其餘至少 90%之水不溶性部份並無任何用途，成爲廢料而被丟棄。本研究初步結果顯示，靈芝子實體纖維爲主體的加工廢棄物可加工純化，並製成人工薄膜，而經由初步的動物實驗證明其對大白鼠的皮膚切除傷口有迅速癒合的功能。其主要機轉可能來自靈芝子實體纖維所含的幾丁質及 1，3-glucan 複合體所引發的。此符合 Pruden(1970)發現 poly-nacetylglucosamine (chitin)爲創傷癒合物質，也呼應日本利用螃蟹殼幾丁質作爲人工皮膚創傷被覆材之商品例證。但靈芝子實體廢料比起螃蟹殼幾丁質所製成的同類商品更具有良好的特性：

1、以靈芝子實體纖維爲人工皮膚取代物的創傷被覆材料尚爲先 例，如屬可行則經由真菌培養將不虞匱乏的提供作爲材料來源。

2、靈芝萃取後的廢料仍具有菌絲的結構，不需要像幾丁質一樣的經過溶解抽絲的過程，就可直接編織成多孔性薄膜簡化了製備流

程。

3、靈芝爲傳統使用的藥材，由過去到目前均無有害報導，在使用上的顧慮較少。

4、此爲靈芝的廢料提供另一實用之用途，提高附加價值及環保影響。

貳、實驗方法

一、靈芝多孔性薄膜製備

靈芝經熱水萃取後，其廢渣經磨碎後以 95%酒精處理除去脂溶物，經 1N NaOH 加熱水解後再以 10% 次氯酸鈉漂白洗淨得到純白色絲狀物，將其脫水後經冷凍乾燥成爲多孔性薄膜，此成品經溼熱滅菌法消毒後即可使用。

二、癒傷效果評估

本實驗所使用的動物爲雄性 Wistar 大白鼠重量約爲 300 至 400 克均單獨飼養於籠中防止互相啃咬，飼料和飲水均自由取用，由於大白鼠活動力極強爲防止傷口受損，使用運動用彈性繃帶固定保護傷口。麻醉方法則採用 ketamine 35mg/kg 和 pentobarbital 12mg/kg 溶於蒸餾水中，分別實施腹腔注射但避免事先混合產生沈澱，而麻醉後以電動理髮刀剔除大白鼠背部和腹部毛髮，然後仿照 Kaufman 的做法，於大白鼠背部位於第十二對肋骨以下和薦骨髁關節以上之部位，以 22 公分見方的版模，在離中線一公分的位置，用油性筆繪出成鏡相的兩正方形。再以外科方式用手術刀、剪刀和鑷子沿繪線的內沿切除此兩塊皮膚，切除方法類似 Smahel 於其研究

中所用之方法，深度達到 panniculus carnosus 再以紗布和 0.9% 鹽水清洗傷口之血漬後，隨機地分配一傷口予靈芝多孔性薄膜覆蓋處理，另一傷口則以紗布處理，爲對照組。兩者均以 0.9%鹽水溼潤敷料，以使敷料和傷口能緊密的貼合，傷口經敷料處理後，須再以彈性繃帶環繞給予保護性固定，以防止脫落，在完成手續後，將大白鼠置回籠中觀察約一小時左右。待恢復後，放回動物房，並於術後的第四、八、十二、十六、二十天予以觀察測量傷口面積。於觀察期間，傷口敷料若有脫落，則立即給予敷料並重新固定。

三、傷口面積之計算

以水性鋼珠筆用透明紙將傷口形狀描繪下來，經適當處理後，再以掃描器將之轉換成圖形檔，再以軟體 Imagine pro 計算傷口之面積。

參、結果

經觀察計算結果，發現蘇老師靈芝組與曾老師靈芝組在第四、八、十二、十六、二十天均顯著較紗布組爲小，見圖 1、圖 2、圖 3、圖 4、圖 5。

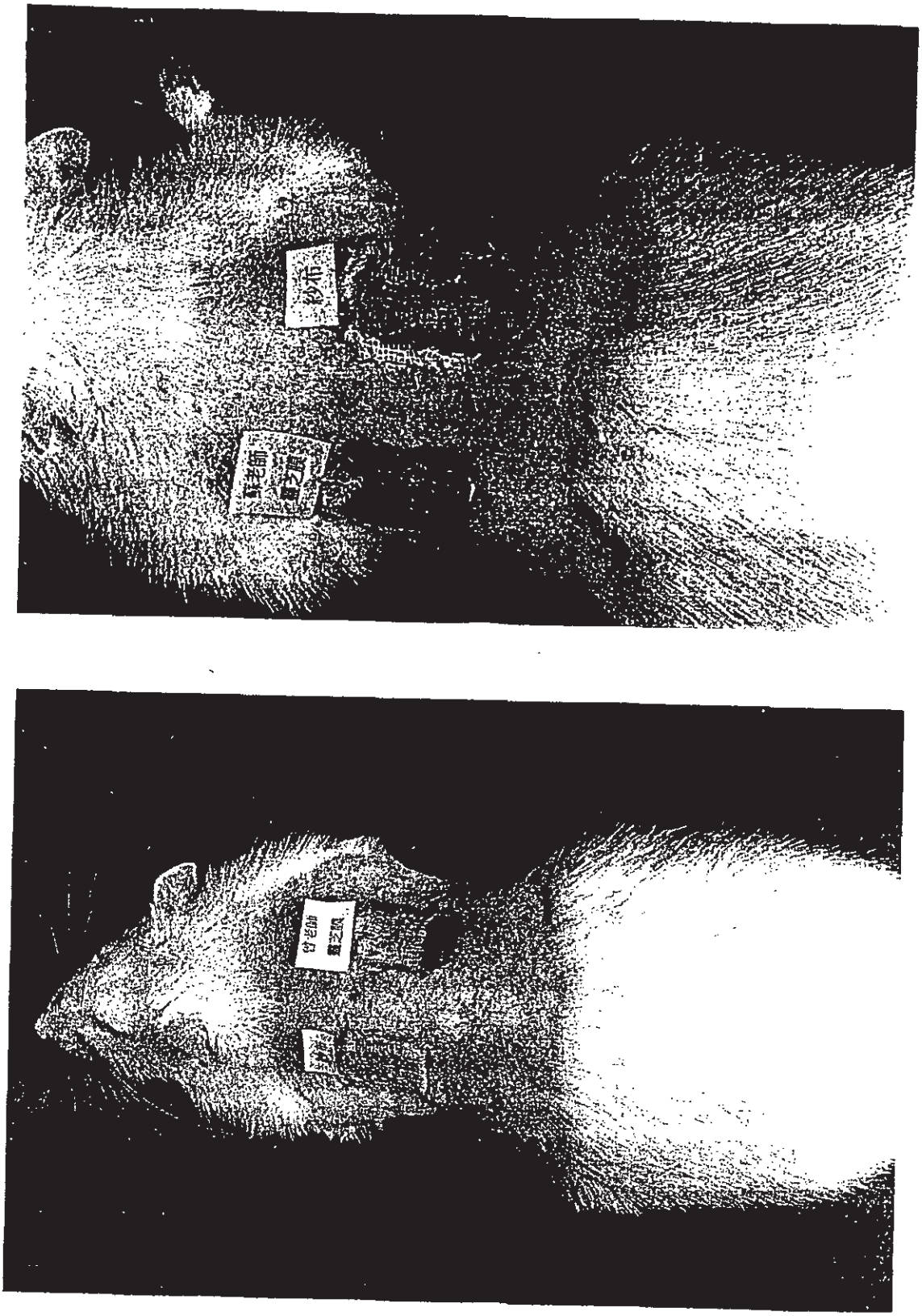


圖 1、白鼠創傷試驗，於第四天之癒合狀況，傷口覆被材以紗布爲控制組，靈芝 Saccharchitin（蘇老師）及靈芝溶解成膜（曾老師）爲實驗組。

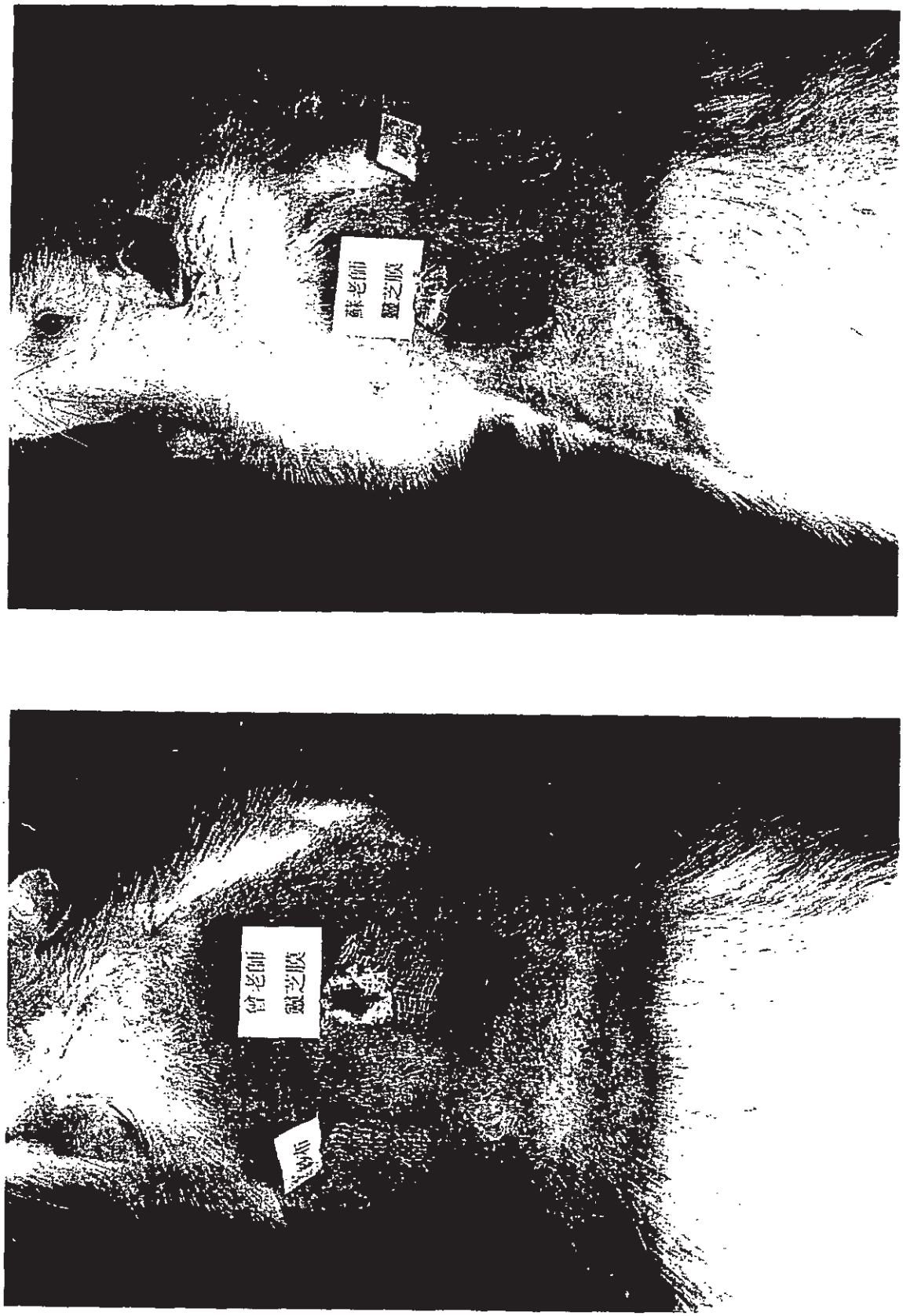


圖 2、白鼠創傷試驗，於第八天之癒合狀況，傷口覆被材以紗布爲控制組，靈芝 Saccharchitin（蘇老師）及靈芝溶解成膜（曾老師）爲實驗組。

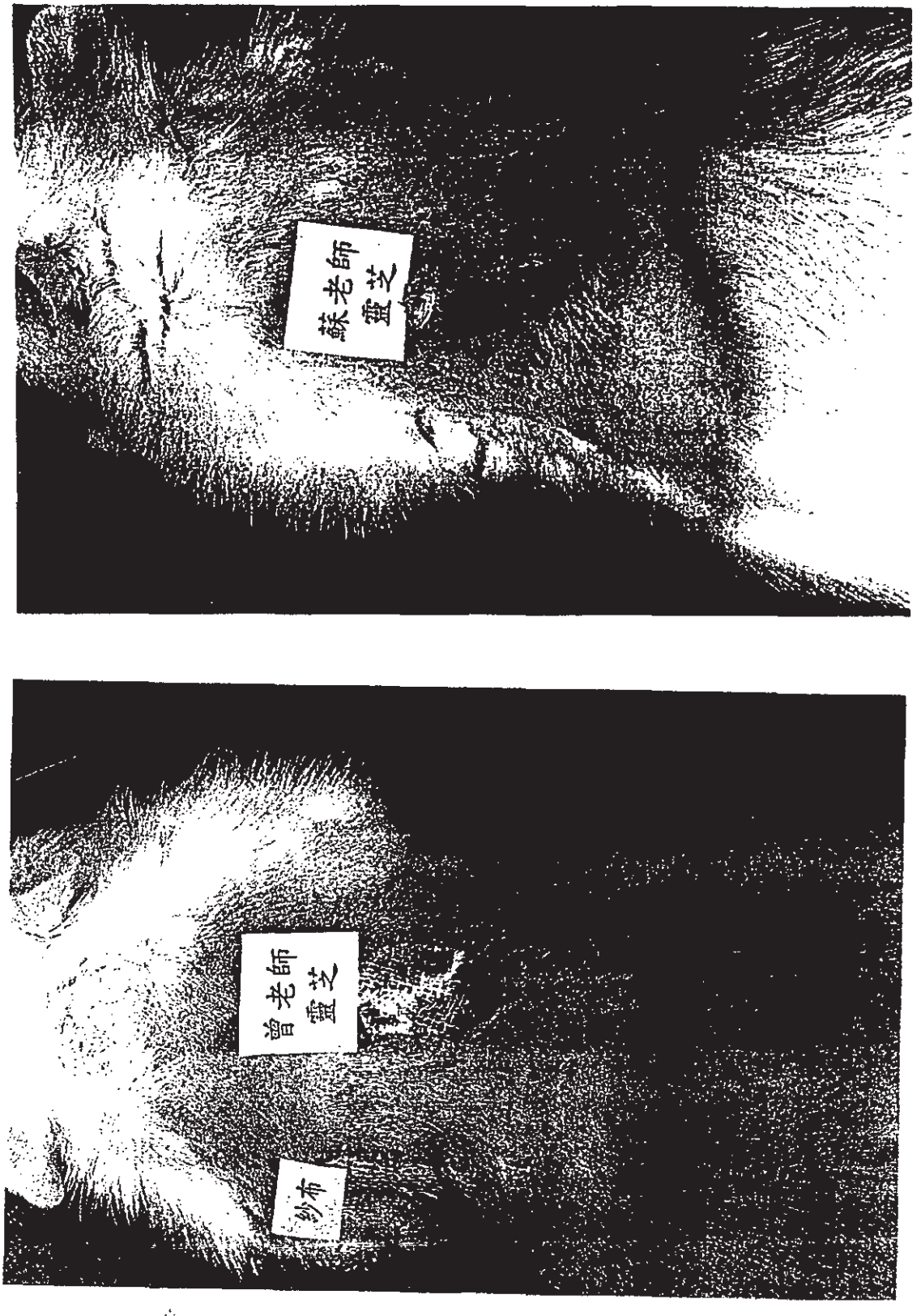
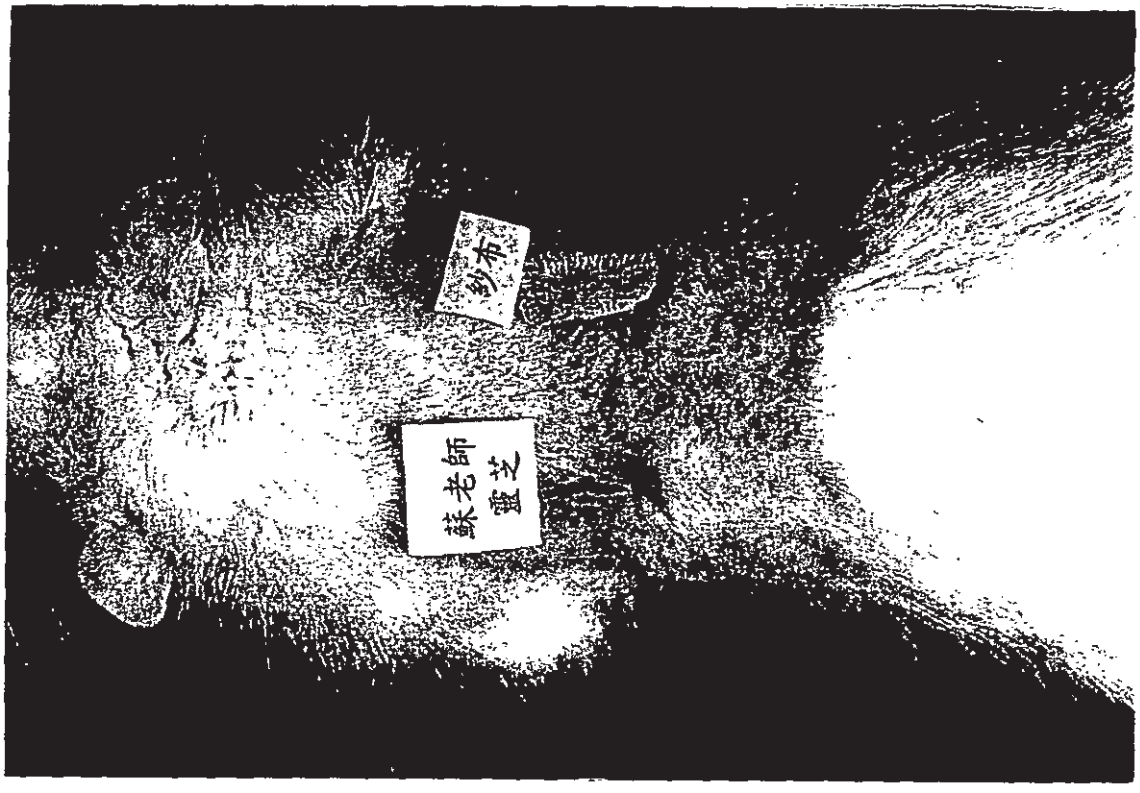


圖 3、白鼠創傷試驗，於第十六天之癒合狀況，傷口覆被材以紗布爲控制組，靈芝 Saccharchitin（蘇老師）及靈芝溶解成膜（曾老師）爲實驗組。



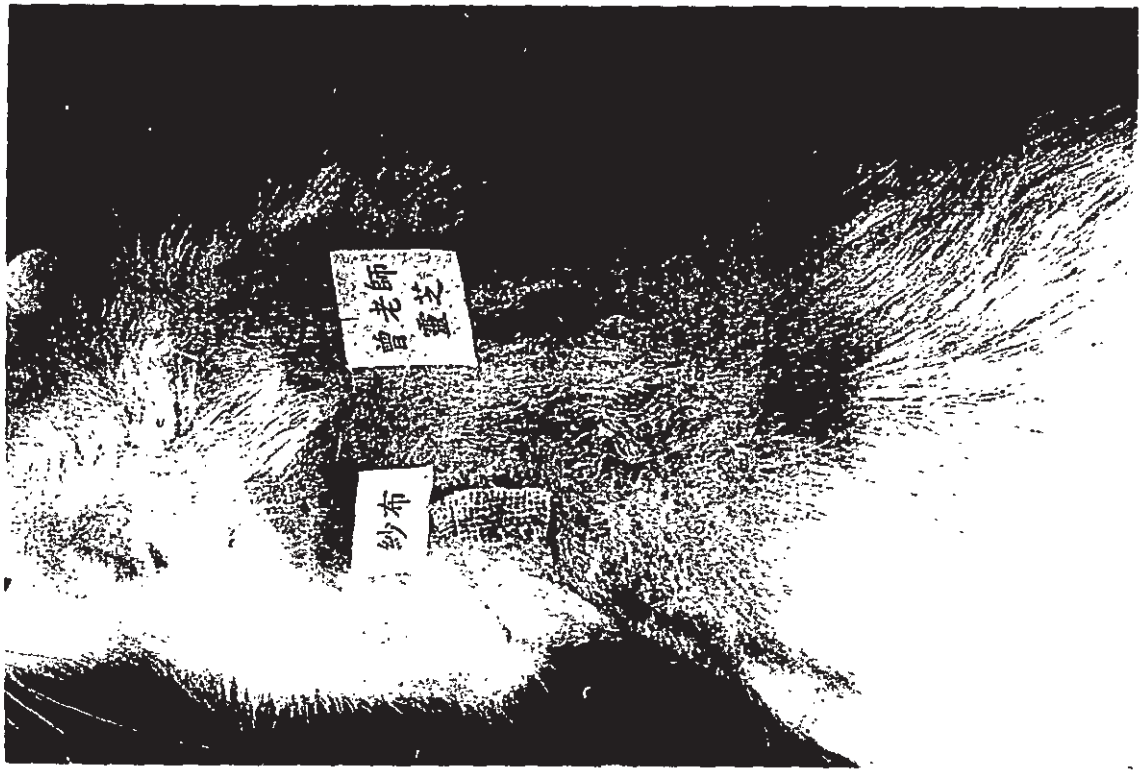
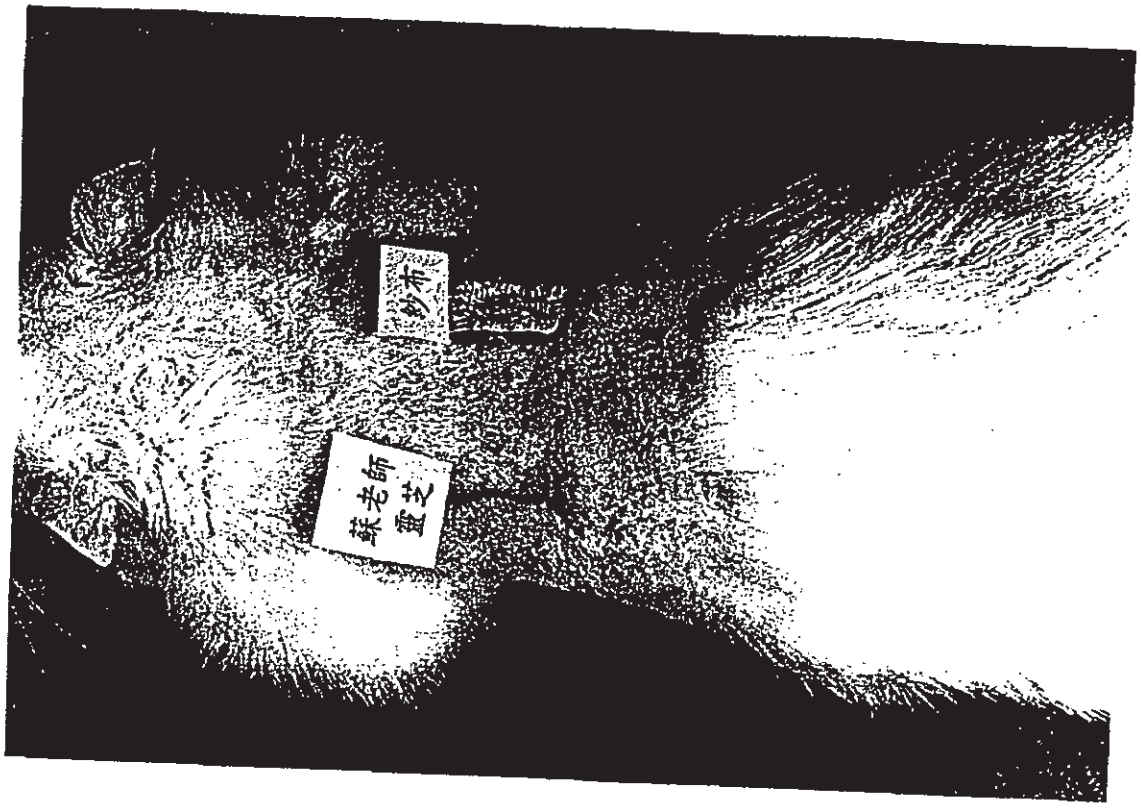


圖 4、白鼠創傷試驗，於第十二天之癒合狀況，傷口覆被材以紗布爲控制組，靈芝 Saccharchitin（蘇老師）及靈芝溶解成膜（曾老師）爲實驗組。



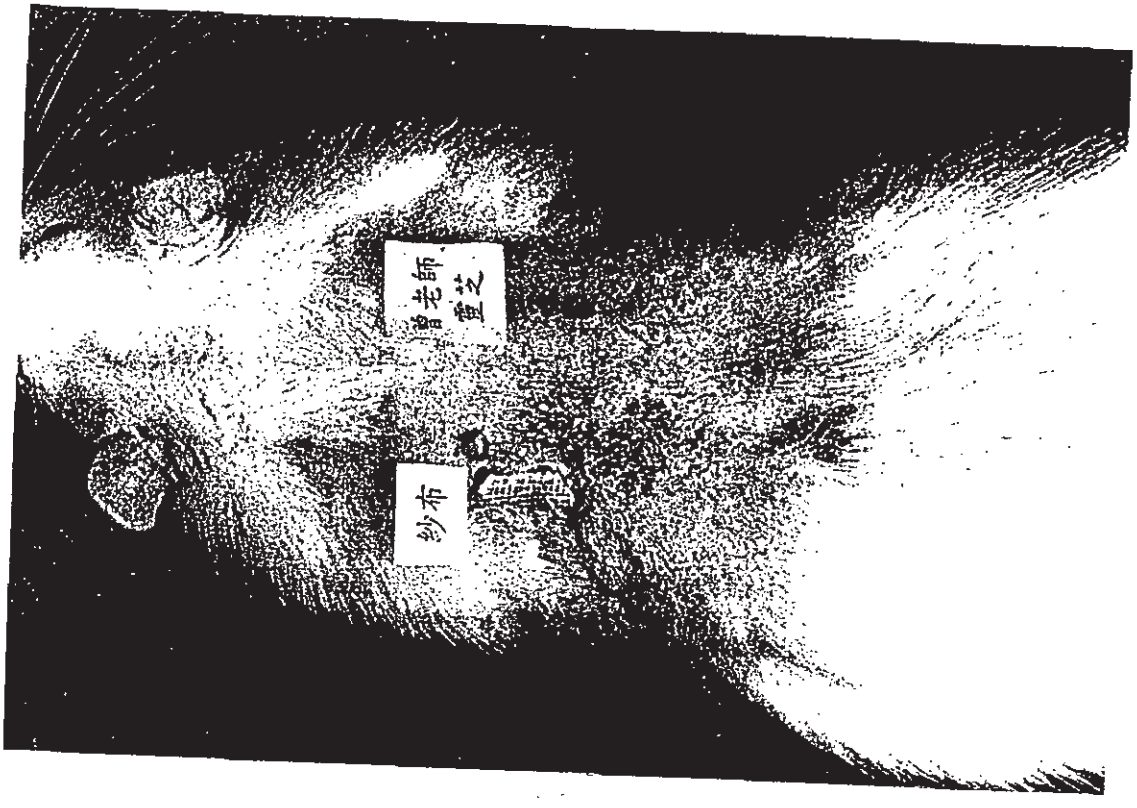


圖 5、白鼠創傷試驗，於第二十天之癒合狀況，傷口覆被材以紗布爲控制組，靈芝 Saccharchitin（蘇老師）及靈芝溶解成膜（曾老師）爲實驗組。

子計畫-2（胡俊弘）

壹、實驗方法

一、 樣品的製備

靈芝膜以粉碎機磨成碎粉取 0.03 克，以 12N HCl 加熱至 100°C 處理 5 小時後，再加入適量去離子水以超音波振盪，最後定量至 10ml，並以針筒過濾器(Pore Size: 0.4μm)過濾後得到。

二、 HPLC 的條件

管柱：CHO-620

流速：0.5 ml/min

溫度：900°C

壓力：33kg/cm^2

靈敏度：32X

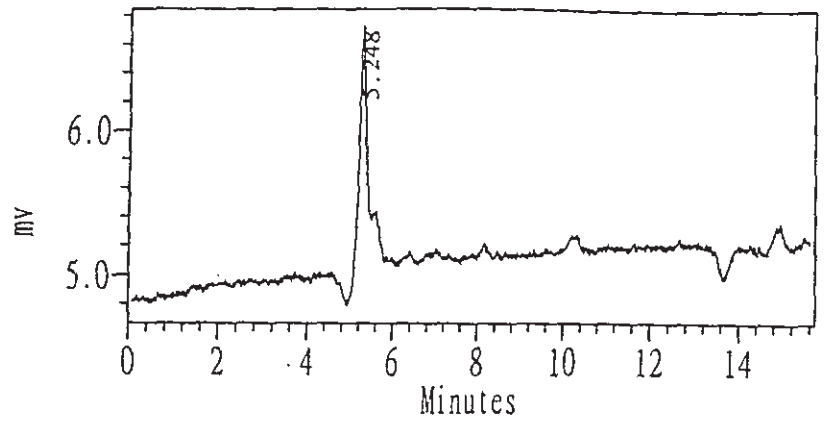
檢量線：D-glucosamine

三、 結果

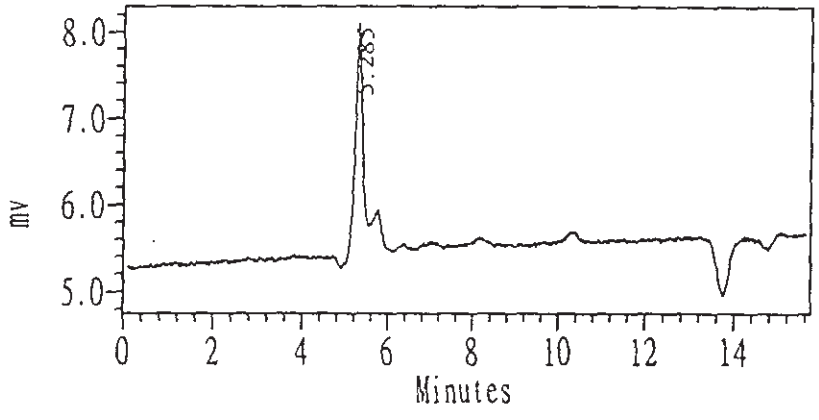
編號		學　名	比例
1	36042	Ganoderma tsugae	37.9
2	36064	Ganoderma sp.	34.1
3	36124	Ganoderma lucidum	52.3
4	36144	Ganoderma lucidum	57.7
5	36146	Ganoderma resinaceum	41.5

6	36147	Ganoderma resinaceum	35
7	36152	Ganoderma mirabile	34.5
8		Ganoderma australe	49.4
9	36041	Ganoderma lucidum	25.2
10	36049	Ganoderma neo-japonicum	51.7
11	37053	Ganoderma lucidum	48
12	36065	Ganoderma tsugae	47.9
13	36066	Ganoderma australe	30.5
14	37067	Ganoderma fornicatum	51.9
15	36087	Ganoderma subamboinense	47.4
16	36125	Ganoderma licidum	44.2
17	36128	Ganoderma australe	44.7
18	36145	Ganoderma weberianum	39.6
19	36159	Ganoderma pfeifferi	48.7
20		Rhizopus stonifer	51.8

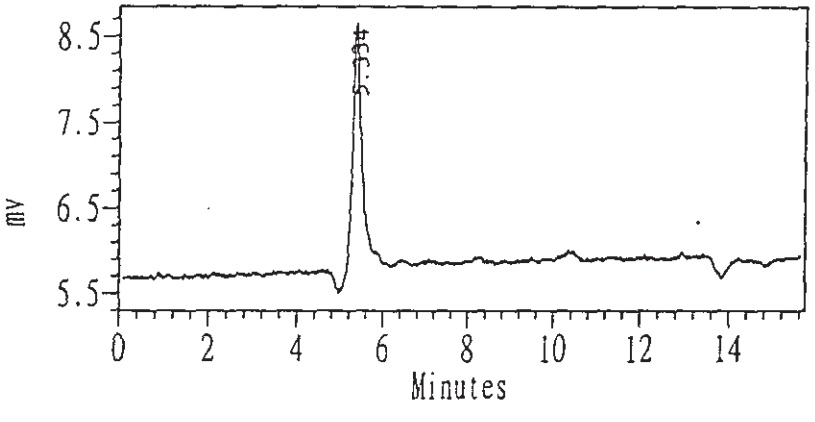
sample1 36042



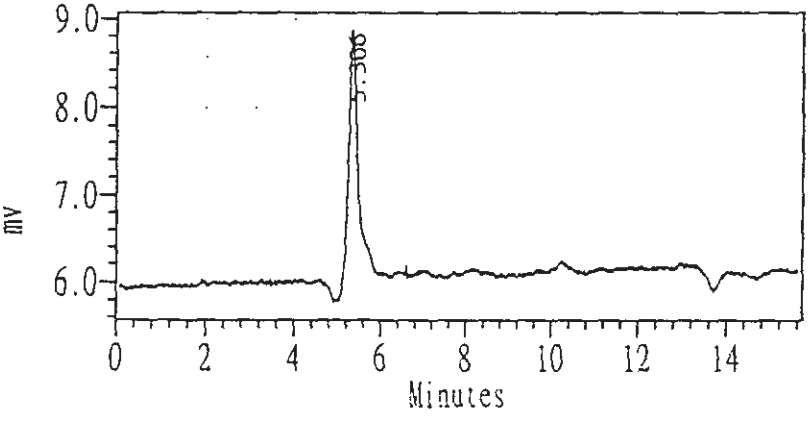
sample2 36064



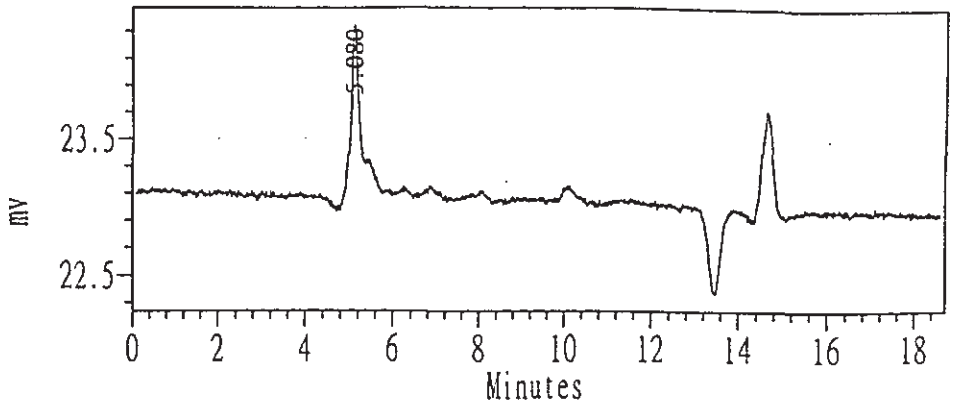
sample3 36124



sample4 36144



sample9 36046



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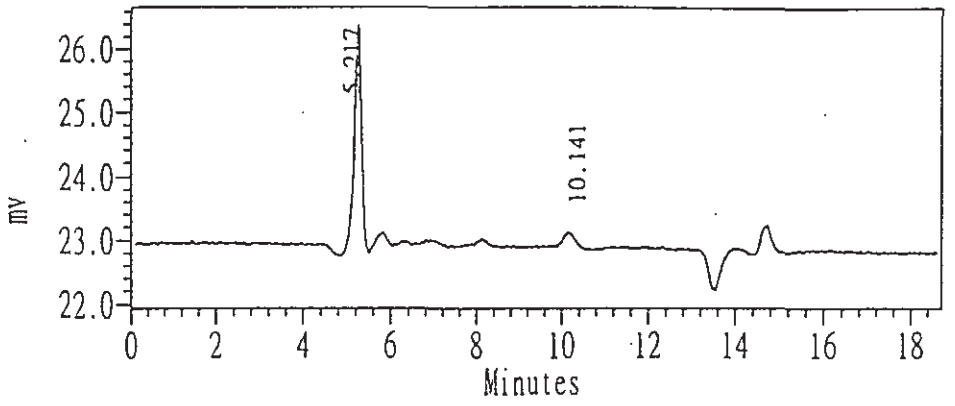

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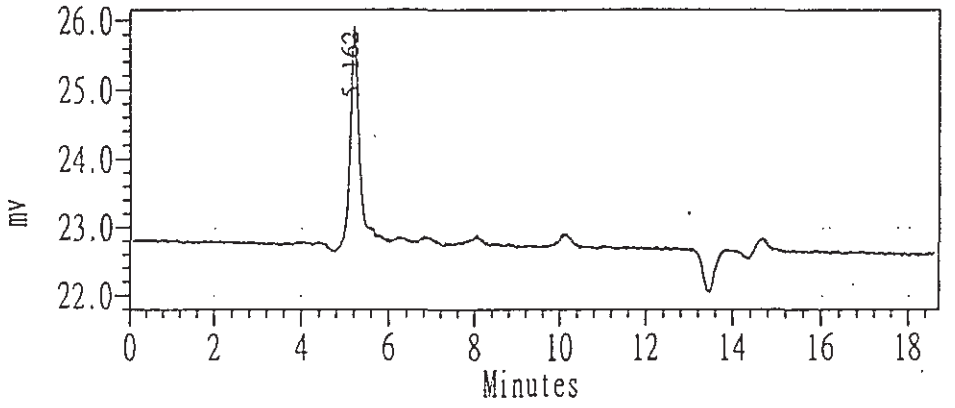
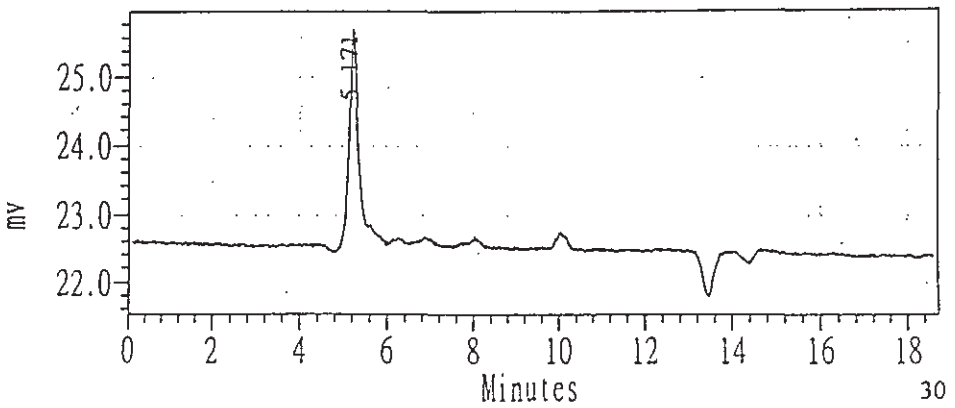


sample12 36065



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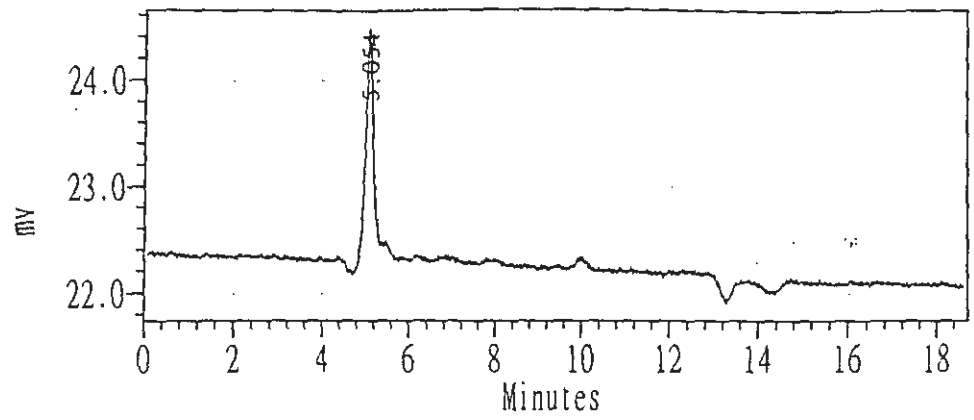

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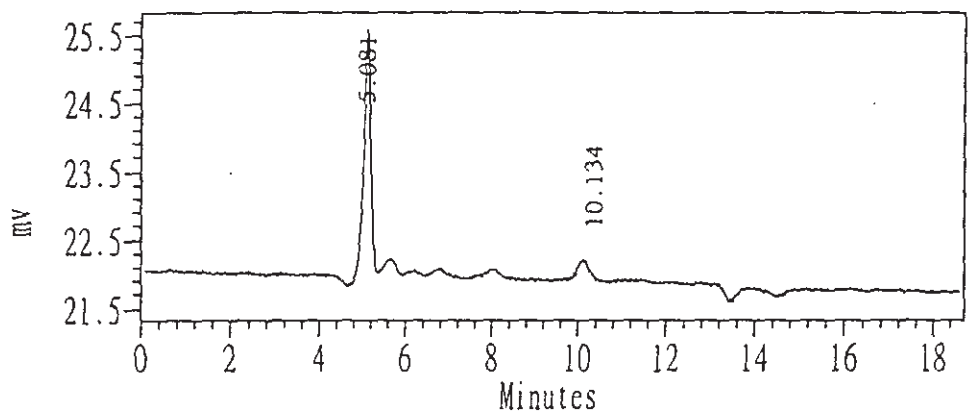
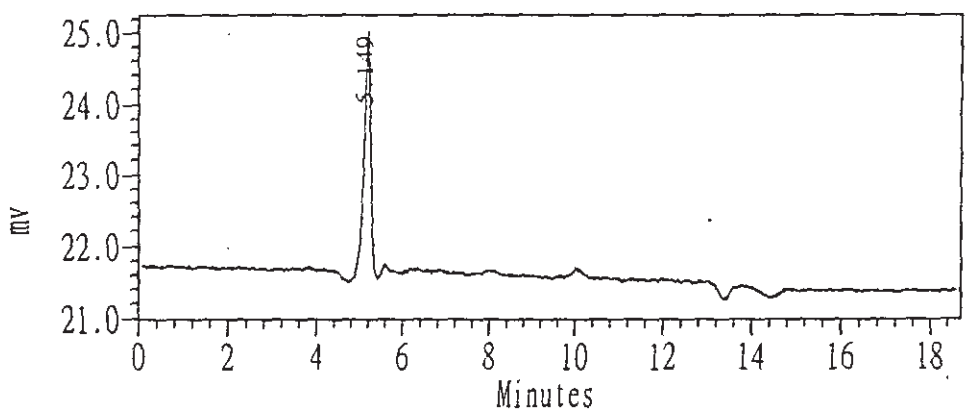
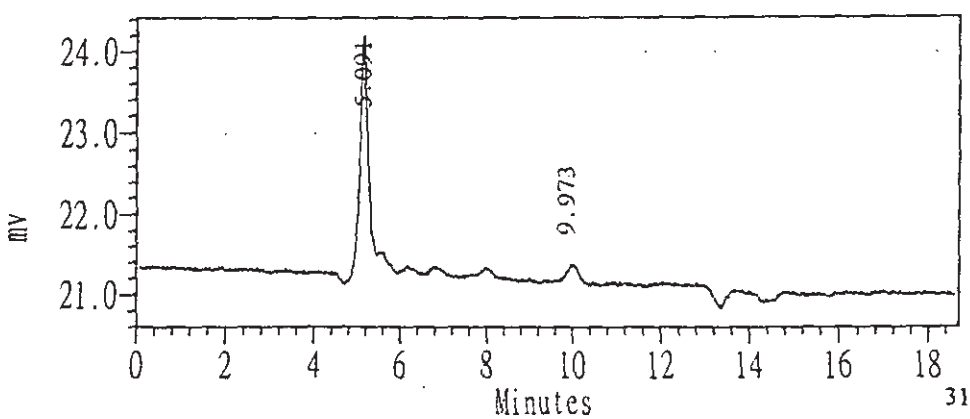


sample15 36081



sample16 36125



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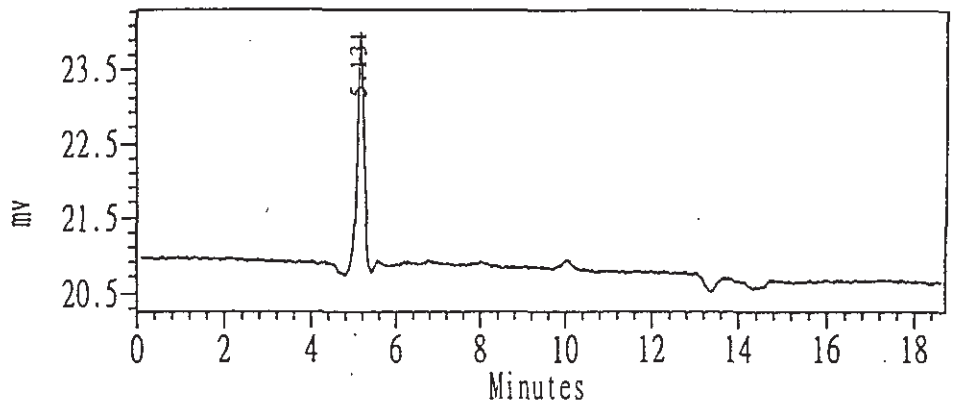

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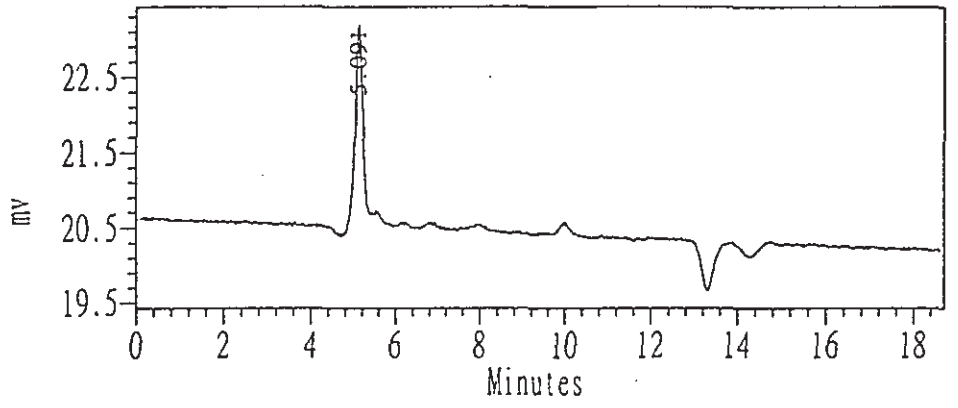

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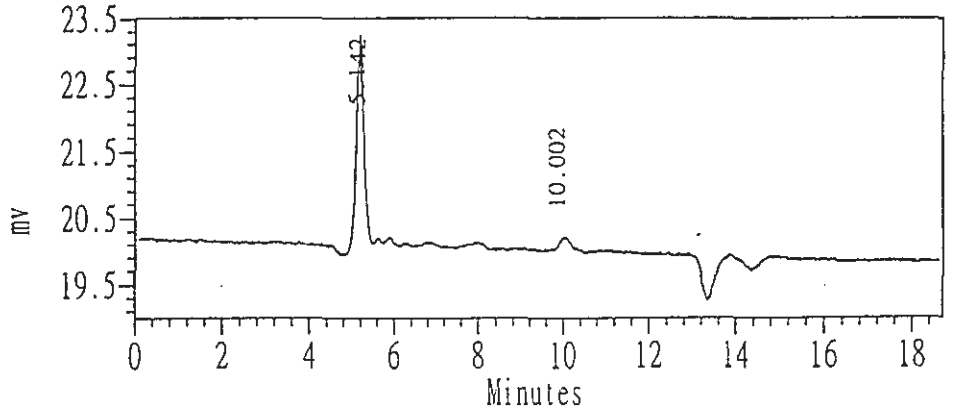
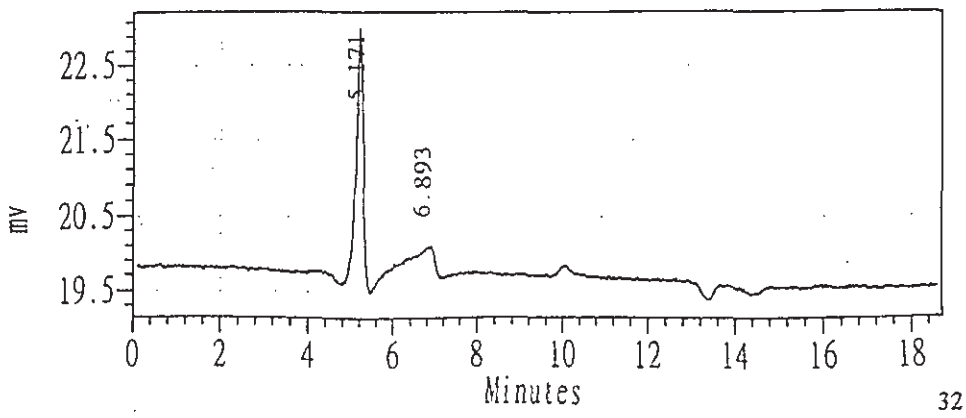
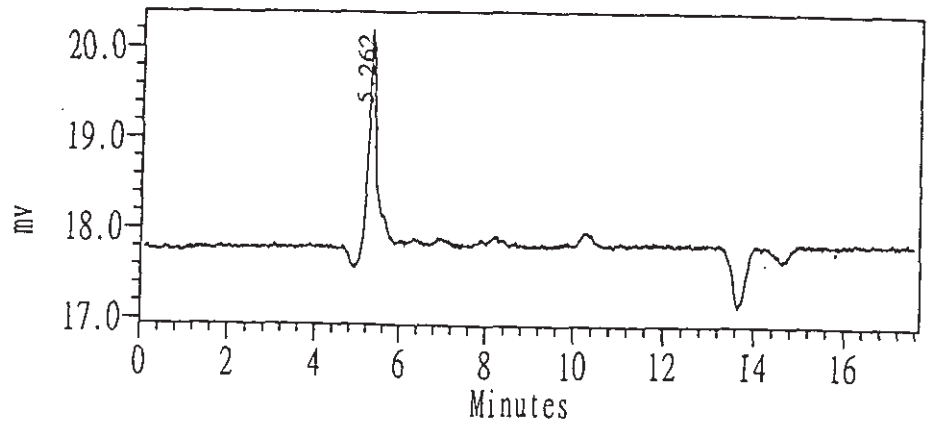


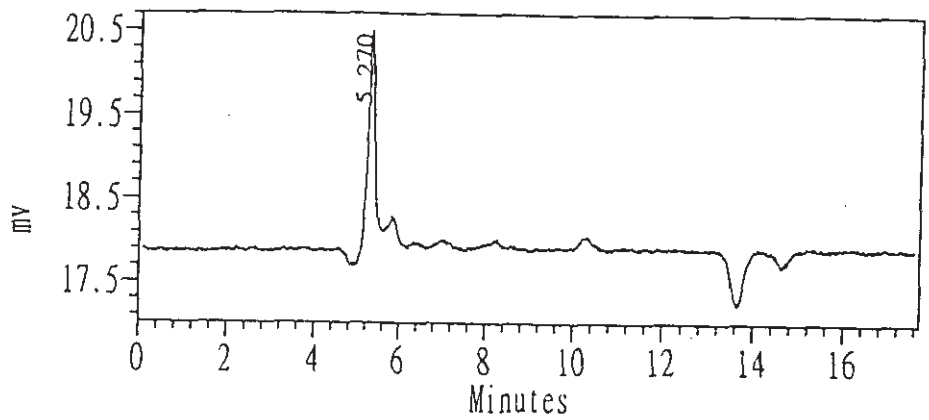
sample20 54



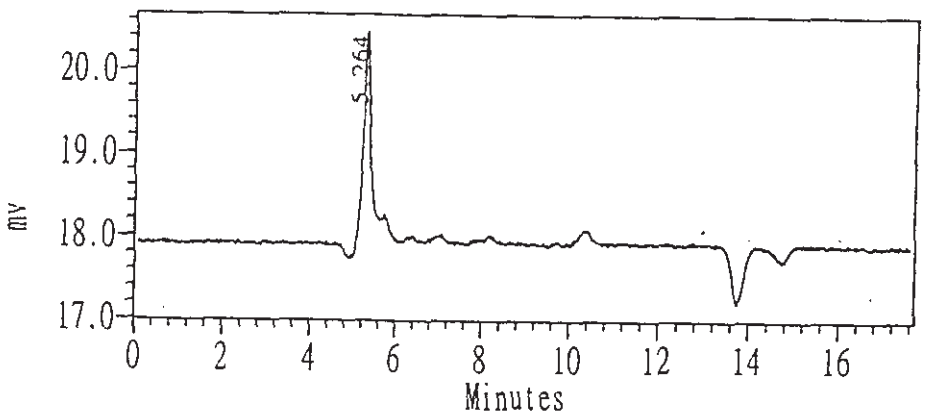
sample5 36146



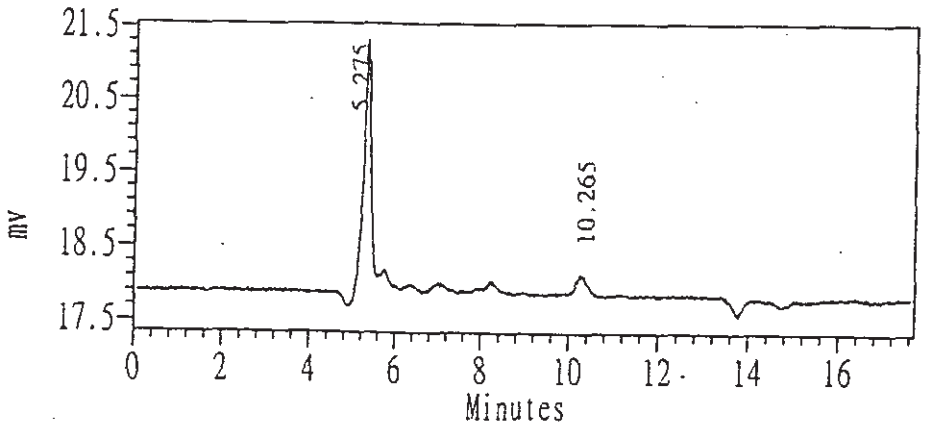
sample6 36147

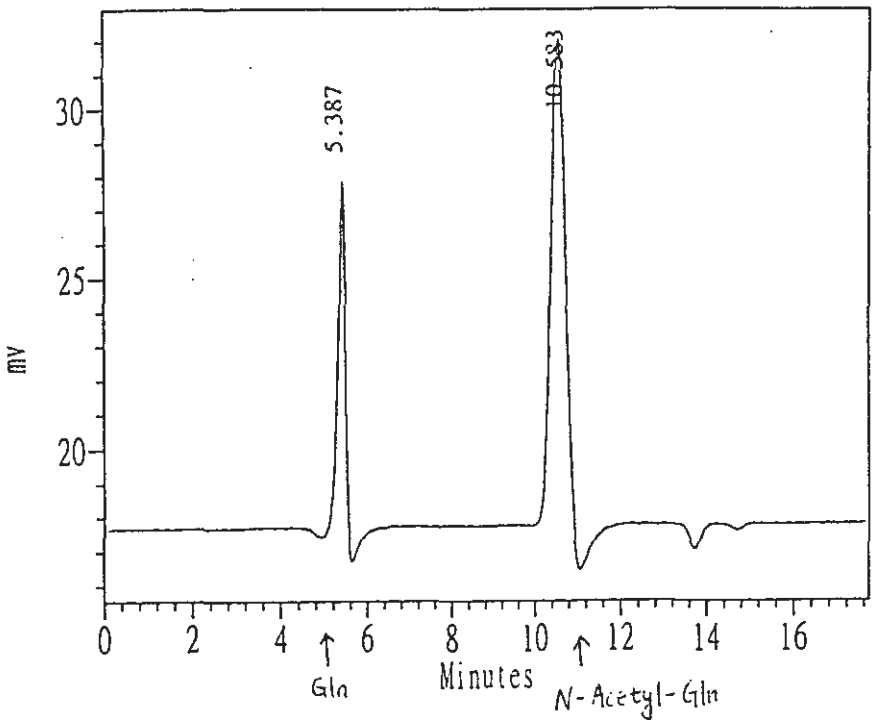


sample7 36152



sample8 [illegible]




30
25
20
mV
5.387
10.583
0
2
4
6
8
10
12
14
16
Gln
Minutes
N-Acetyl-Gln

子計畫-3（曾厚）

壹、研究目的

靈芝是一種重要的醫學材料取代物，最近在生物上的應用以及商業市場上當作健康食物已經被廣泛的探討。通常在除去水可溶解的部分後，靈芝所留下的不可被水溶解的菌絲纖維(超過 90%)是沒用的。菌絲纖維的結構較其他大多數的多醣類來的複雜。菌絲纖維是由β-1,3Glucan 和 poly(N-acetyl- β-D-glucosamine)所組成。β-1,3Glucan 和 poly(N-acetyl- β-D- glucosamine)都可被人體吸收代謝。之前，Pudden 已經證明 poly(N-acetyl- β-D-glucosamine)在創傷治療上有顯著的效果，並且指出由 poly(N-acetyl- β-D- glucosamine)水解所產生的 oligo(N-acetyl- β-D-glucosamine)具有較高免疫賦活性(adjuvant capacity)。我們之前已經確定從菌絲纖維製備出來的皮膚包附物在創傷的醫療上有加強治療的效果。生物的降解，吸收，免疫賦活性等性質以及改善創傷治療使得菌絲纖維成爲一種較其他合成材料吸引人的生物醫學材料。綜合以上的發現，我們預期菌絲纖維可以備用來當作皮膚取代物的生物醫學材料。然而目前爲止並沒有一個適合的溶劑可以溶解菌絲纖維使我們得以繼續材料的製程。

由於菌絲纖維的化學性質與殼糖相似,以我們對殼糖的了解，應該可以找出一些可供菌絲纖維溶解的溶劑。除了在含有氨基酸溶劑中加入鋰鹽以及醇類中加入鈣鹽之外，

大部分之前找到可溶解殼糖的溶劑往往會造成殼糖分子量的降解。在氨基酸溶劑中加入鋰鹽包括三種系列：LiCl/DMAc(N,N-dimethylacetamide)，LiBr/DMAc 以及 LiCl/DMAc/NMP(N-methyl 2-pyrrolidone)其中以 LiCl/DMAc 最常被使用。到目前爲止在醇類中加入鈣鹽的方式尚未被應用在作爲殼糖的溶劑。在最近文獻中 MMNO(N-methyl morpholine N-Oxide)以成爲一種有潛力的多醣溶劑。在這個子計畫中，我們想要找到一個可以溶解菌絲纖維的溶劑系。爲了達到這個目的，我們將從 LiCl/DMAc，鈣鹽/醇類以及 MMNO 中選取一個適合的溶劑。在找到適合的溶劑後，我們將可以從菌絲中萃取我們需要的成分，在改善了牠的物理性質及生物吸收性後可以作爲皮膚的包覆物或取代物。

從靈芝中可以製備高分子量的菌絲,在體外的實驗中包括菌絲膜的物理化學性質，及細胞毒性和細胞遷移的改善，在這個子計畫中我們要確保菌絲膜的所有性質可以改善創傷治療。從我們先前在老鼠皮膚的研究中顯示由不能被水溶解的靈芝殘渣中所製備出來的皮膚包附物在創傷治療上有顯著的效果。我們所得到的結果與 Pruden 在 1970 所得到的結果一致，也與日本某一研究團體發現從甲殼類的外殼萃取的殼糖可以成膜作爲人工皮膚相似，這個結果是令人興奮的，因爲在靈芝的使用中原本沒有用的部分(不被水溶解的部分)具有應用在臨床醫療的經濟價值的潛力。在這實

驗中，我們已經擁有殼糖化學的一些有關溶解、修正及分析的經驗。由於菌絲纖維的性質類似殼糖，所以我們可以找到菌絲纖維的溶解系統，並且製備出多種靈芝菌絲。

貳、研究方法

我們試圖找到菌絲纖維的溶解系統，並且避免菌絲纖維在溶解的過程中產生分子量的降解三種溶劑系統包括 LiCl/DMAc，$CaCl_2 \cdot 2H_2O$/MEOH 和 MMNO 將是我們嘗試的方法，LiCl/DMAc 在溶解菌絲纖維將是第一個目標，菌絲膜可被製造出來，同時量測該膜的物理性質，接著，我們亦將探討有 casting 的情況下對膜的化學、物理性質是否有影響。

$CaCl_2 \cdot 2H_2O$/MeOH 對菌絲纖維的溶解能力也將被探討。菌絲纖維膜將以溼菌絲纖維旋轉的原理來製備。這種製程條件下所生產的膜，其物理化學性質也將被測量。綜合三、四個子計畫，細胞的培養及其毒性的測試和細胞的遷移效應也都是注意的結果。

從靈芝粹取出來的 Sacchachitin 由第一子計畫提供 Tokura 曾對於純化的菌絲材料的應用原理有所研究，一些靈芝菌絲在 135°C 中持續 3hrs 被 50%的 NaOH 乙醯化，LiCl 在使用前需在負壓下且超過 185°C 乾燥，DMA 和 DMF 需在氮氣中減壓不加入 CaH 進行蒸餾並儲存在超過 Lynde Type 4Å molecular sieve 中。至於其他化學藥品就不必被純化了。

一、LiCl 在 DMAc 中的溶解：

在一個燒瓶中加入 35ml 的 DMA、2.5g 的 LiCl，這個混合物需在 70°C 下加熱一小時，並攪拌持續一小時，然後將此混合物冷卻至室溫並加下純化的菌絲 0.25g，然後再一次加溫到 70°C 持續十二個小時，並且攪拌。溫度再降到 0°C~5°C 之間並攪拌，在這溫度持續二小時，最後再加溫到 70°C 並攪拌，如此幾小時後便可得到澄清的均質溶液。

二、 LiCl/DMAc 中菌絲纖維的溶解：

將 6wt%(將對於 DMAc)的靈芝菌絲放入 LiCl/DMAc 中，加熱至 70°C 並攪拌，持續 18 小時，降溫至 0°C~5°C 之間並攪拌，在這溫度持續二小時，最後再加溫到 70°C 並攪拌，持續四小時，重複二小時，四小時，共升降溫四次。

三、 成膜：

1. 入離心管中進行離心，離心機設定，轉速 3000rpm，10 分鐘。
2. 離心後，將上層液收集，下層不溶解物質乾燥後收集，爲成膜所用，以便日後分析。經過四次升降溫之後，將三孔燒瓶中的混合液倒。
3. 將步驟 2 中之上層液一半利用回旋蒸發器加以濃縮(將多餘的 DMAc 抽出)，一半加入殼糖。
4. 準備三個塑膠盆分別盛入 Acetone，Ethonal 及水，準備四個玻璃板，自製的 casting 器具。

5. 將步驟 3 中製備的濃稠液倒在玻璃板上，再以固定方向做 casting，以增強膜的力學性質。
6. 將 casting 後的濃稠液連同玻璃板放入有 Ethonal 的塑膠盆中，並前後搖動玻璃板，約莫三分鐘。
7. 將步驟 6 之 sample 取出，放戶有 Acetone 的塑膠盆中，並前後搖動玻璃板，約莫三分鐘。
8. 將步驟 7 之 sample 取出，放入有水的塑膠盆中，靜置約莫一分鐘。
9. 此時膜已大致不附著在玻璃板上，將膜放入大水盆中靜置。
10. 重複步驟 6~9
11. 以清水不斷的沖洗剛成型的膜，以去除 LiCl 及 DMAc。
12. 再以蒸餾水清洗數次。
13. 以蒸餾水浸泡數小時。
14. 取適量浸泡膜的蒸餾水，滴入 AgNO3，檢驗膜是否仍有 LiCl 的存在。
15. 以不銹鋼將膜攤平。
16. 自然風乾。

三.1 黏度測定：

測定菌絲成乙醯化菌絲，溶液的黏度可以得知菌絲纖維是否在溶解的過程造成了分子量的降解。且爲測量在不同濃度的 LiCl/DMAC 中靈芝殘渣的溶解度與其黏度的關係，可方便日後由黏度的測定得知靈芝殘渣在 LiCl/DMAC 中的溶

解度。其測定流程如下：

1 X%LiCl/DMAC(X=1,2,3,4,5)+6%靈芝殘渣，攪拌並加熱。

2 升降溫 4 次

3 離心，取上層液爲 Sample

4 取適量的 Sample 作再沈，求出溶解度

5 以同濃度之 LiCl/DMAC 稀釋，做出 5 各不同溶解度的 Sample

6 以黏度計測量各 Sample 的黏度

7 計算各 Sample 的本質黏度

8 作黏度與濃度的關係圖（圖一、二）

而所得溶液再沉的方法如下：

1. 取適量的 Acetone(與待測的靈芝萃取液體積比爲 7:1)倒入燒杯並以攪拌子攪拌。

2. 緩緩倒入靈芝萃取液。

3. 將步驟 2 的溶液離心(需將步驟 1 的燒杯以 Acetone 洗滌乾淨並一起離心)。

4. 將上層液到掉。

5. 取 methanol 加入步驟 4 的離心管中(含有離心後的下層物)，並攪拌，使其成爲懸浮液，再加以離心。

6. 同樣地，將上層液到掉。

7. 最後，加入清水，重複步驟 5，6。

五、NMR 測定：

將製備的膜，經攪碎機打成粉末狀交付貴重儀器中心

測試，以便得知菌絲纖維膜之的化學結構。

六、強力測試：

1. 測量膜的平均厚度。

2. 將膜剪成 10mm 寬的 sample 後置上拉力試驗機(Shimazu Autograph)。

3. 輸入膜的資料，設定以 0.5mm/min 的速度來作拉力測試。

4. 在測試儀器的 load cell 上，貼上標籤紙，防止 load cell 將膜夾破。

5. 記錄拉力測試中的 max-load、max-displacement、max-stress 及 max-strain。

七、細胞毒性測試(MTT Assay)

1. 每毫升 105 的纖維母細胞，以每 well 加入 0.2 毫升，在二氧化碳濃度 5% 及溫度 37°C 下，培養 24 小時。

2. 以 5 毫升 DMEM 培養液浸泡實驗材料一天。

3. 隔日，各取 0.1ml DMEM 培養液分別加入 well 內。

4. 每個 well 加 0.01 毫升 MTT salt 反應 4 hr。

5. 每個 well 加入 0.1 毫升 solubilization 4 hr。

6. 24 小時浸泡。

7. ELISA reader。

8. recorder(nm)。

肆、結果與討論

1.LiCl 在 DMAc 中的粘度表現

2.菌絲纖維在 LiCl/DMAc 中的粘度表現

3.成膜

4.結構鑑別

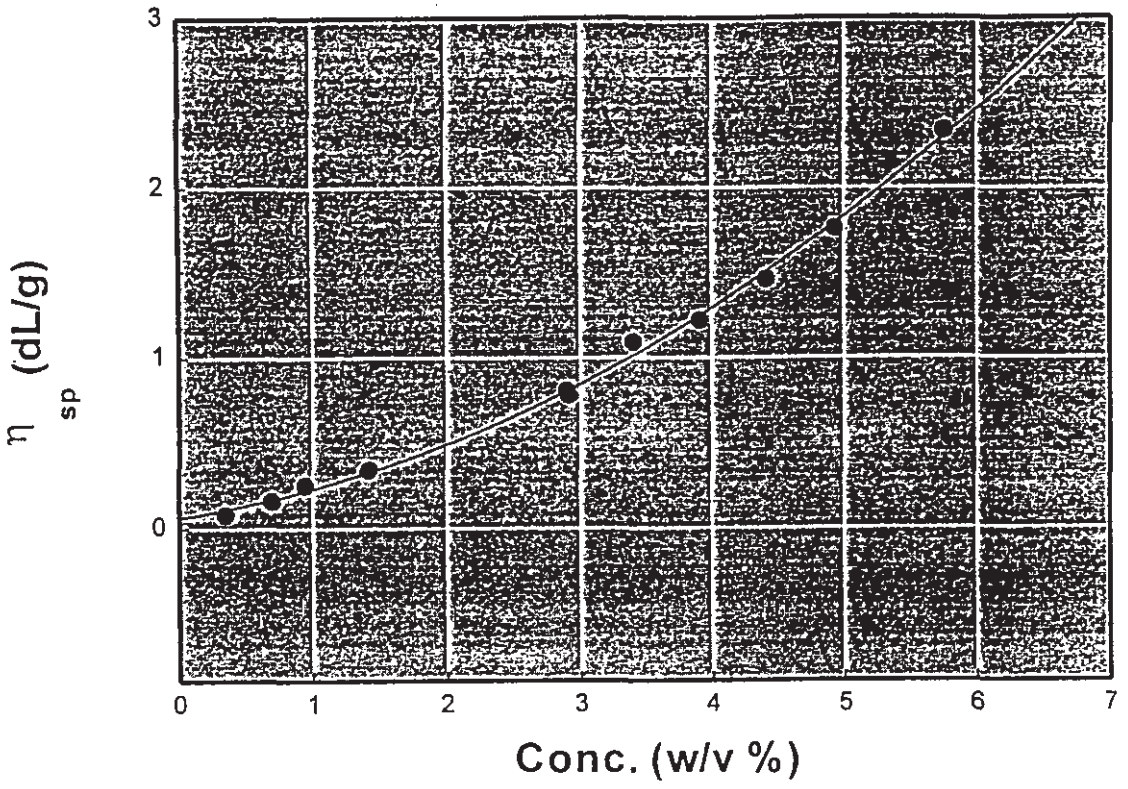
5.強力測試

當使用者要將菌絲纖維膜用在傷口上時，膜的力學強度是重要的考量。太脆會使膜太早破裂，然而太結實的膜則較難敷在傷口上，也就是說較弱的膜無法支持關節的運動，太強的膜則因爲無法吸收運動時的能量而使得膜容易移位。收集這些基本的必要訊息及其菌絲纖維膜的最佳力學強度，對於日後製造產品的精製過程有很大的幫助，對於使用菌絲纖維膜的人也較有效果。菌絲纖維膜需被製備成 0.2mm 厚×100mm×10mm 的片狀膜以便作靜力測試，sample 的兩端都要貼上 3M 的貼布以保護 sample，再將 sample 的一半浸入 37°C 的食鹽水中，另一半則曝露於空氣中持續二十四小時，二十四小時後時 sample 用 AGS-D series，Shimadzu，Co，Japan 的機器作拉力測試，設定 1mm/min 的位移速率直到 sample 斷裂爲止，顯示電腦記錄的圖表及、分析數據在膜的製程中，膜被滾製成薄薄的一層，並在高溫下烘乾，這滾動與烘乾的過程大大地影響了膜的力學性質(包括 tensile strength、shear strength 以及 toughness)爲了得到更多有關菌絲纖維膜在不同 strain rate 以及不同

溫度下的性質，動態測試是必須做的。DMA 通常被用來測定、模擬菌絲纖維膜製程中的性質分析。這些資訊大大地幫助產品的製造，就像如何改變開口大小，網狀纖維的拉、滾過程，及其在乾燥時有適當的溫度等。更進一步說，如果菌絲纖維膜被使用在身體上常動的部分，(例如：關節皮膚或是敷在嘴巴上的膜等)將會遭受拉力改變 strain rate，同時溫度亦爲有所變化。DMA 對於預測類似生物體運動造成菌絲纖維膜性質的變化非常有用。

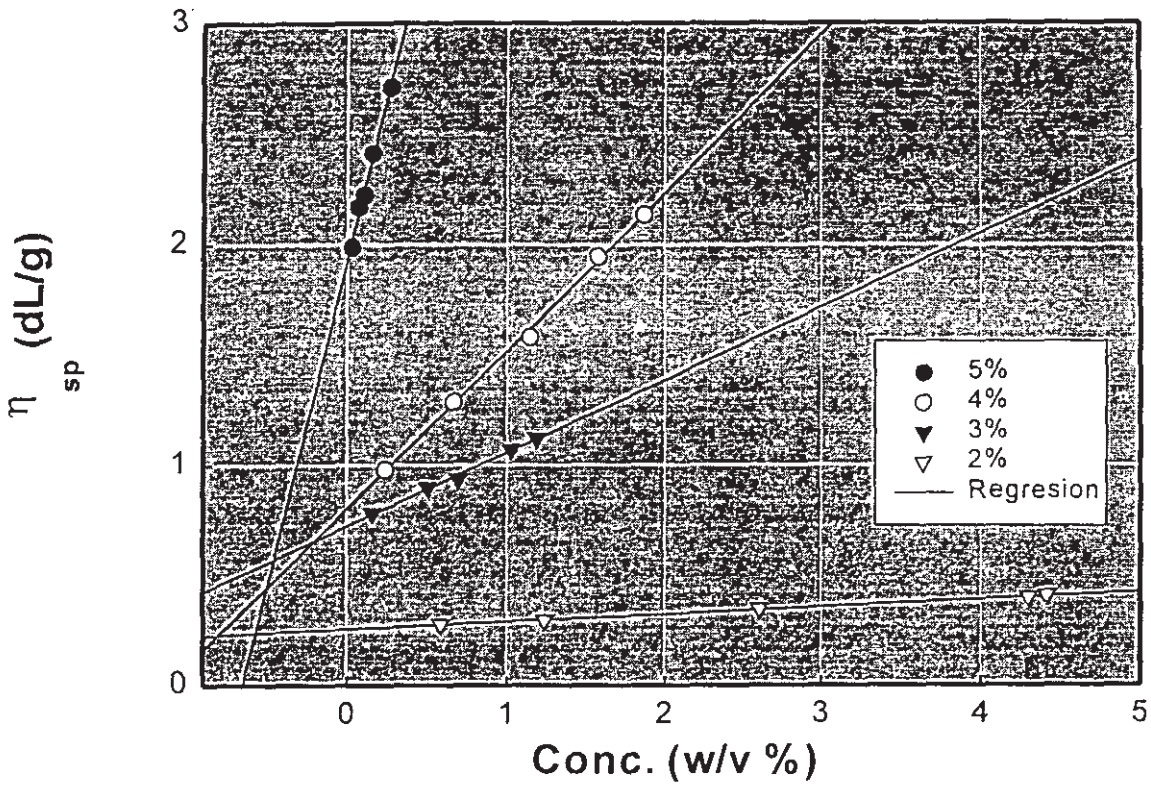
Sample 的製備類似靜力測試，chamber 的溫度設定在 37°C，strain frequency 設定在從 0.1sec^{-1}到 10sec-1，每個間距 0.5sec^{-1}，重複 DMA 的測定改以固定 0.1 的頻率，改變溫度從 0°C 到 75°C，每次間隔 0.5°C。

6. 細胞毒性測試



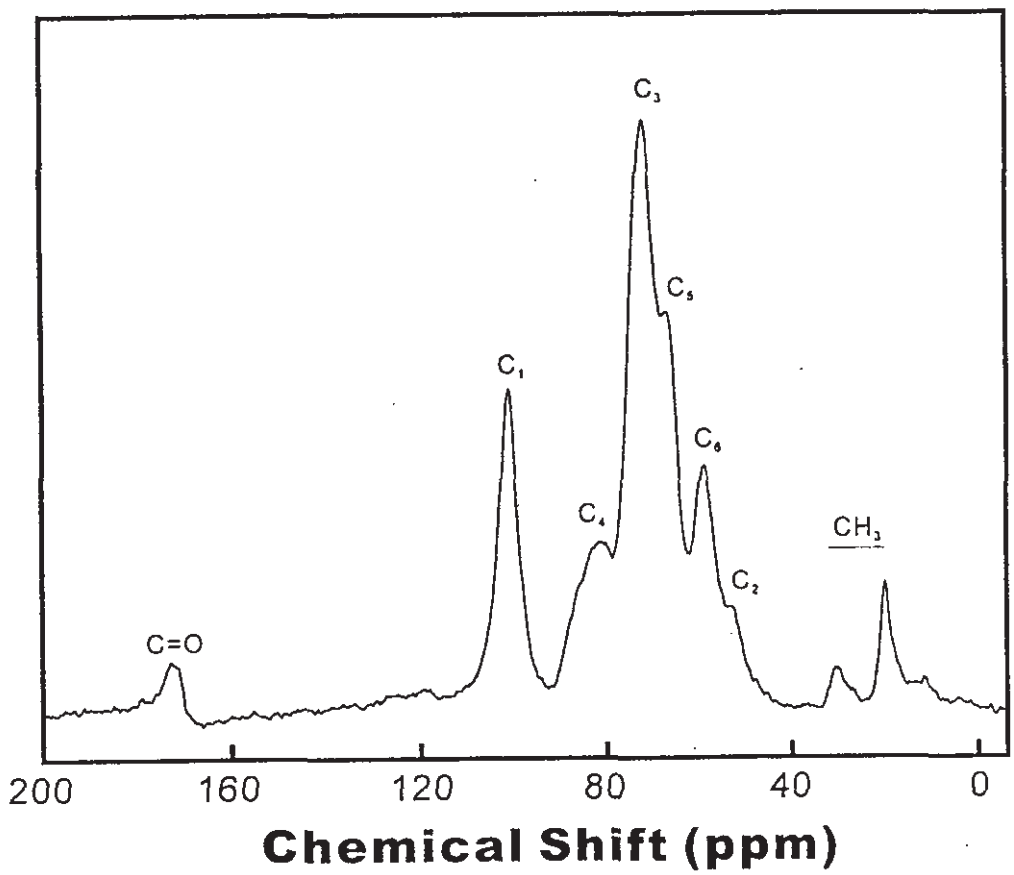
圖一. LiCl在DMAc中的粘度函數圖

LiCl濃度 (g/dL)	比粘度 (dL/g)
0.35	0.08
0.70	0.16
0.95	0.26
1.42	0.34
2.90	0.81
2.91	0.78
3.40	1.09
3.90	1.22



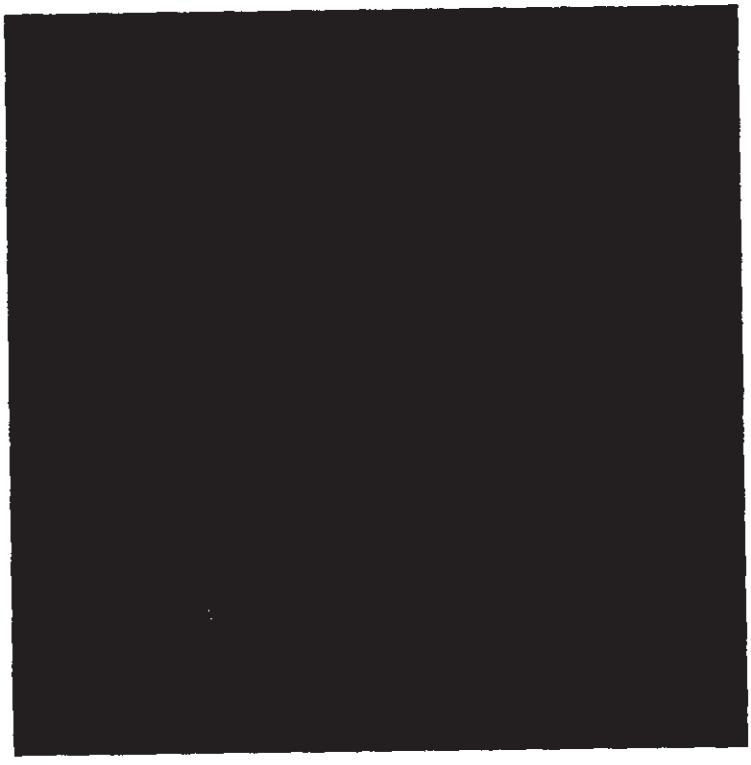
圖二. 菌絲纖維LiCl/DMAc中的粘度函數圖

Conc. of LiCl/DMAc (w/v %)							
5%		4%		3%		2%	
0.03	1.99	0.24	0.98	0.16	0.78	0.59	0.28
0.07	2.17	0.67	1.29	0.51	0.90	1.24	0.29
0.11	2.22	1.14	1.58	0.70	0.94	2.61	0.35
0.15	2.41	1.57	1.95	1.03	1.07	4.30	0.40
0.27	2.71	1.86	2.13	1.19	1.12	4.41	0.41



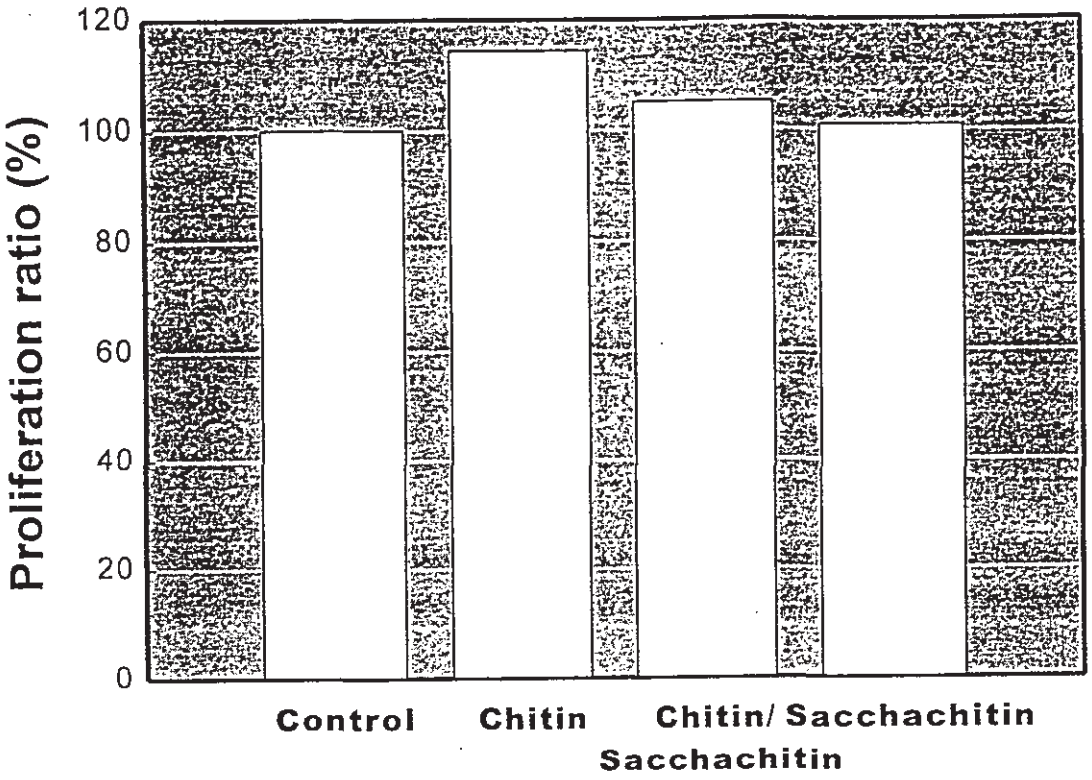
圖三. 菌絲纖維的^{13}C CP-MAS NMR光譜圖

(A)



(B)

圖四. (A)菌絲纖維膜,
(B)加入殼糖的菌絲纖維膜
之實體照片



圖五. 各種膜的毒性測試比較圖

子計畫-4（蔡郁惠）

壹、實驗目的

運用靈芝皮來當作皮膚的取代物，用於燒燙傷或割傷的病人皮膚上，藉以提昇傷口部位的癒合能力，是一種新的生物醫學材料的應用。故在將靈芝皮當成生物醫學材料運用在臨床醫療之前，靈芝皮可能對於物的細胞有毒性作用(cytotoxicity)，對動物體產生免疫性(immunogenicity)、以及過敏反應(allergic response)等有待更進一步的探討。本實驗評估了由靈芝的殘餘物所製成的靈芝皮敷用於傷口上，當作皮膚的取代物時，對纖維母細胞的生長與毒性分析(cytotoxicity)；利用靈芝皮懸浮液，來觀察其對老鼠皮膚是否會產生過敏反應（allergic response）及免疫性(immunogenicy)，進而瞭解靈芝皮是否對纖維母細胞之生長有影響以及對動物體是否有不良副作用。

貳、實驗材料及方法

一、實驗動物:

重 400±50g 的 wistar 老鼠。

二、實驗材料:

靈芝皮(Sacchachitin)由蘇慶華老師提供。另外兩種純靈芝膜及靈芝加幾丁質膜則由曾厚老師所提供。

三、實驗方法:

（一）評估靈芝皮對於纖維母細胞的細胞毒性分析

1. 細胞的初級培養(Primary cell culture):

纖維母細胞（fibroblast）的初級培養是由八週大裸鼠的皮膚而來。

2. 纖維母細胞的毒性的測試:

利用 MTT (0.5 mg/ ml) 來測量靈芝皮對於所培養的細胞其生長的效果。

(1) 測試細胞的培養：

將 2 ml 的 2 x 10^4 cells / ml 的纖維母細胞，加入至 6-well plate 的每個 well 中，在 37℃，含有 5% CO_2 的培養箱中培養 24 小時。

(2) 靈芝皮對細胞毒性的測試：

將靈芝皮懸浮液以 medium 稀釋成 1%，0.5%，0.1%，0.05%，0.01%等濃度後，加入靈芝皮懸浮液（2 ml）至每個 well 內，再放入培養箱與細胞作用三天。之後加入 MTT（2 ml）至每個 well 內，再培養 4 小時後，加入 2ml 的 DMSO 溶解紫色結晶，利用分光光度計以 570 nm 的波長來讀取吸光值。

（二）、觀察靈芝皮懸浮液對老鼠皮膚所產生的過敏反應的測試

利用皮下注射方式將靈芝皮懸浮液打入已剃毛的老鼠背上，觀察靈芝皮懸浮液對老鼠的皮膚是否會產生一些過敏現象，例如:起疹、發紅、起水泡等。

（三）、觀察靈芝皮懸浮液對於老鼠的免疫性(immunogenicity)

1. 抗原的產生:

將 1 ml 之靈芝皮懸浮液 (1%) 與 complete Freund's Adjuvant 以 1:1 的比例混合，利用皮下(subcutaneous)注射的方式，打入老鼠的體內。待 3 星期後，再將 1 ml 之靈芝皮懸浮液與 incomplete Freund's Adjuvant 以 1:1 混合的第二次抗原打入老鼠的體內，隔 3 個星期後，再打入第三次的抗原後，隔 7 天及 14 天，再抽取老鼠的血液以進行下面的實驗。同時以 PBS，Freund adjuvant only 做 negative control，並以 type I collagen 及 type II collagen 以同樣方法來做 positive control。

將收集到的血液用 2,000 x g 離心 10 分鐘後，取上清液所得到的血清 (serum) 來進行 ELISA 的試驗。所收集到的血清則儲存在-20℃。

2. 酵素聯結免疫吸者分析法(Enzyme-Linked Immunosorbent Assay，ELISA)

將靈芝皮懸浮液溶在 0.1M Carbonate 內 coating 在 96-well plate 之 wells 內中(放置於 4℃ 一天後)，加入 10% milk 在 37℃ blocking 30 分鐘，用 PBST 洗 3 次，加入用 10% milk 稀釋的血清，放置於 4℃一天，用 PBST 洗 3 次後，加入 10% milk 在 37℃ blocking 30 分鐘，再用 PBST 洗 3 次，加入用 10% milk 稀釋的次級抗體 (secondary antibody)（1：2000），再於 37℃ 作用 1 小時後，用 PBST 洗 3 次後，加入 TMB，待呈色後，加入 4N HCl 來終止反應。利用 ELISA reader 來讀取 OD_{450} 的吸光值。此實驗以打過 collagen type I 及 collagen type II 之血清當 positive control，以施打 PBS 的血清當作 negative control。

參、實驗結果

一、靈芝皮(Sacchachitin)對傷口的癒合能力

以同一隻老鼠的左右兩側各切除 2 x 2 cm^2 大小面積的皮膚，並同時在其兩側分別覆蓋上靈芝皮（Sacchachitin）或紗布(cotton gauge)，並在 24 小時，3 天，7 天，14 天，18 天，21 天分別測量靈芝皮與紗布所覆蓋的傷口面積大小。結果發現在第 7 天左右，覆蓋靈芝皮的傷口面積比覆蓋紗布的傷口明顯地縮小很多，而且在第 21 天左右，覆蓋靈芝皮的傷口幾乎已完全癒合，而覆蓋紗布的傷口則是尚未完全癒合，由此可知靈芝皮的確具有促進傷口癒合的能力。

二、不同之靈芝皮對裸鼠纖維母細胞之毒性分析

1. 靈芝皮(Sacchachitin)的分析：

將裸鼠之纖維母細胞處理 1%，0.5%，0.1%，0.05%，0.01% 等不同濃度之靈芝皮懸浮液三天後，加入 MTT 反應 4 小時，利用 DMSO 溶解所產生的紫色結晶，在 570 nm 波長下測其吸光值，並將處理靈芝皮懸浮液的細胞之吸光值除以只加入培養基的細胞之吸光值，若值小於 100%既表示在某濃度下的靈芝皮懸浮液對纖維母細胞具有毒性。所得之結果如圖 1-(a) 所示，可知當靈芝皮懸浮液在小於 0.1%（w/v）的濃度之下，對於裸鼠的纖維母細胞並無毒性。

2. 靈芝膜加幾丁質的分析：

將裸鼠之纖維母細胞處理 1%，0.5%，0.1%，0.05%，0.01% 等不同濃度之靈芝膜加幾丁質懸浮液三天後，加入 MTT 反應 4 小時，利用 DMSO 溶解所產生的紫色結晶，在 570 nm 波長下測其吸光值，並將處理靈芝膜加幾丁質懸浮液的細胞之吸光值除以只加入培養基的細胞之吸光值，若值小於 100%既表示在某濃度下的靈芝皮懸浮液對纖維母細胞具有毒性。所得之結果如圖 1-(b) 所示，可知當靈芝皮懸浮液在小於 0.1%（w/v）的濃度之下，對於裸鼠的纖維母細胞並無毒性。

3. 純靈芝膜的分析：

將裸鼠之纖維母細胞處理 1%，0.5%，0.1%，0.05%，0.01% 等不同濃度之純靈芝膜懸浮液三天後，加入 MTT 反應 4 小時，利用 DMSO 溶解所產生的紫色結晶，在 570 nm 波長下測其吸光值，並將處理靈芝皮懸浮液的細胞之吸光值除以只加入培養基的細胞之吸光值，若值小於 100%既表示在某濃度下的靈芝皮懸浮液對纖維母細胞具有毒性。所得之結果如圖 1-(c) 所示，可知當靈芝皮懸浮液在小於 0.05%（w/v）的濃度之下，對於裸鼠的纖維母細胞並無毒性。但是當靈芝皮（Sacchachitin）與靈芝加幾丁質膜對於纖維母細胞之毒性相比較時，純靈芝膜對於纖維母細胞的傷害程度並不如前兩者。

三、靈芝皮對老鼠的過敏反應之探討

將 1%（w/v）之靈芝皮懸浮液以皮下注射方式打入老鼠體內，並在 24 小時以及 7 天觀察老鼠的皮膚，結果如圖 2 所示，箭頭處表示打入靈芝皮懸浮液而突起之處，圖 2-a 表示打入靈芝皮懸浮液後 24 小時老鼠皮膚的外觀，圖 2-b 顯示打入靈芝皮懸浮液後 7 天所觀察到的老鼠皮膚。其結果顯示靈芝皮懸浮液在老鼠體內並無所謂的過敏現象，如發紅等。

四、靈芝皮對於老鼠的免疫性之分析

結果如圖 3 所示，靈芝皮懸浮液在老鼠體內並不會產生抗原抗體的反應，而膠原蛋白（collagen）第一型及第二型則會在老鼠體內引發抗原抗體反應，另外以 PBS 打入老鼠體內亦不會產生抗原抗體反應。而這些所收集到的血清皆爲施打抗原 3 次後（每次間隔 2 個星期打一次），再隔 14 天所收集的血清。

肆、結論與討論

幾丁質爲聚乙醯葡萄糖胺，而且在 1994 年，有學者推論因爲幾丁質是一種帶有正電性的物質，因此可以增加細胞貼附於生長材質上的程度，因而促進細胞生長，但若幾丁質的濃度過高時，幾丁質的正電性會與細胞膜作用，使得細胞受到損害，進而造成細胞的死亡。因此在本實驗室中，三種不同的皮膚取代物，雖其在 0.05%以上的濃度皆會抑制細胞的生長，但是含有幾丁質的兩種靈芝皮（圖 1-a，1-b）比只含純靈芝的膜（圖 1-c）的皮膚取代物對於細胞的抑制作用要來的多，而純靈芝膜

對於細胞的少數抑制作用，可能是所殘留在膜上的漂白劑所造成。

在將靈芝皮液打入老鼠皮下後，觀察其老鼠外觀，並未發現打入的地方有發紅，腫起或起疹的現象，並且也沒有發生潰爛的情形出現，將這些重覆打入靈芝皮液的大白鼠收集血清，用 ELISA 方式測得靈芝皮並未有明顯的免疫性，而 collagen type I 以及 collagen type II 則有很明顯的免疫性發生。

組織轉麩胺酶（tTGase）參與了細胞接合，細胞分化，訊息傳遞，細胞自殺（apoptosis）及傷口修復的調控。有報告指出，在傷口修復期間，細胞內的組織轉麩胺酶會被釋放出來，而且其主要目的是藉由組織轉麩胺酶具有 cross-linking 的功能來穩固傷口。所以當我們能夠知道在傷口癒合期間細胞接合分子(cell adhesion molecules) --轉麩胺酶-- 的變化，便可以推測細胞接合分子在傷口癒合的過程中所扮演的角色。本實驗室利用組織免疫染色的方法，觀察不同天數 t-TGase 的表現有何不同，發現在第 7 天時，t-TGase 表現在覆蓋紗布之傷口的血管壁上，而推測是否與新生血管消失時的 apoptosis 有所關連，所以本實驗室在第 7 天前後來觀察其 t-TGase 的表現，但是目前所遇到的困難是由於血管內的紅血球很多，而影響到結果的判讀。故目前正在極力解決此問題。

將大白鼠背部左右兩側各取下一塊 2 x 2 cm^2 大小的區域，分別覆蓋靈芝皮以及紗布，在第 1 天、第 3 天、第 7 天、第 14 天及第 21 天時取下傷口部份的標本，來觀察靈芝皮對於傷口部

位的發炎反應。在傷口修復的第一階段爲發炎及免疫反應，淋巴球是對免疫反應負責的白血球，而在所有造血原生細胞（hemopoietic progenitors）以及所有白血球（leucocyte）的表面中都有 leukocyte-common antigen (L-CA)的存在，而且這些 L-CA 都是屬於細胞膜上的醣蛋白（glycoprotein），而 L-CA 則包括了有 T200，B 細胞型的 B200，Ly-5 及 CD45。而 L-CA 則是淋巴球主要的細胞膜表面成分。本研究計畫以 CD45 抗體來認識這個 CD45 細胞膜醣蛋白分子，當作在受傷部位其淋巴球表現之標的分子。利用免疫組織化學染色法來觀察在傷口癒合期間，靈芝皮對於傷口所引起的發炎反應情況。但在這一年來，本實驗已試用過不少的抗體，只爲了尋求一支效果最好的抗體出現，以利實驗結果的判讀。現在雖已找到一支較爲適合的抗體，爲其所呈現出來的結果並不好，故目前正在修正實驗的設計，將利用接有螢光物質的 2 次抗體來取代原來接有酵素的 2 次抗體，希望藉由此種改變能夠改善呈色結果太弱而不易判別的困惑了。雖然免疫染色法尚未完成 CD45 的反應，但 H-E stain 確實證實了在傷口癒合過程的第一天就有發炎反應的現象，在傷口部份有 neutrophils 以及 lymphocytes 的出現（如圖 4），而在第七天時，除了可以見到在傷口處有一些較爲緊密的 collagen fiber 所構成的 scar 之外，在這些 scar 外面的表皮層則有角質細胞逐漸生成的現象（如圖 5）。

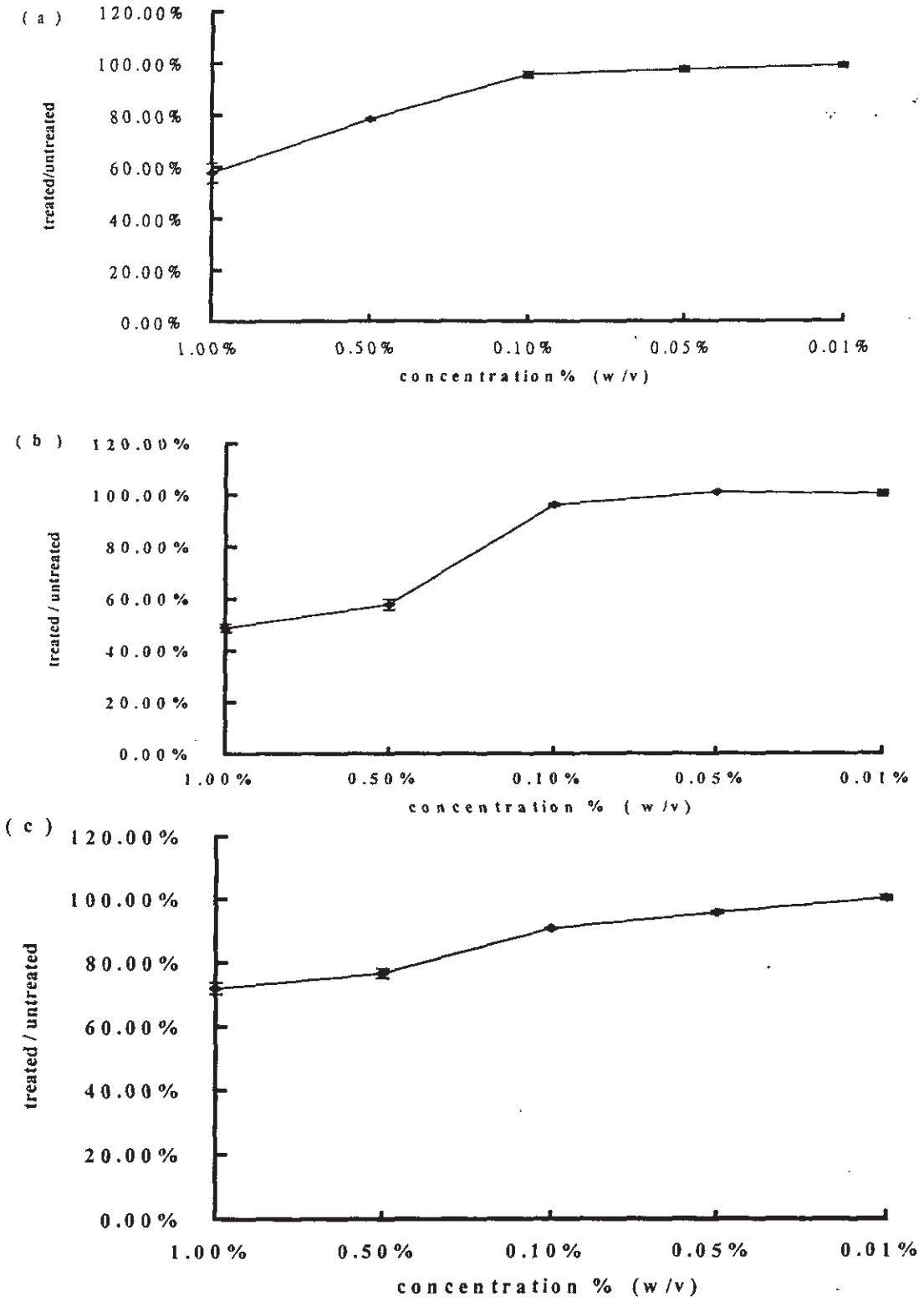
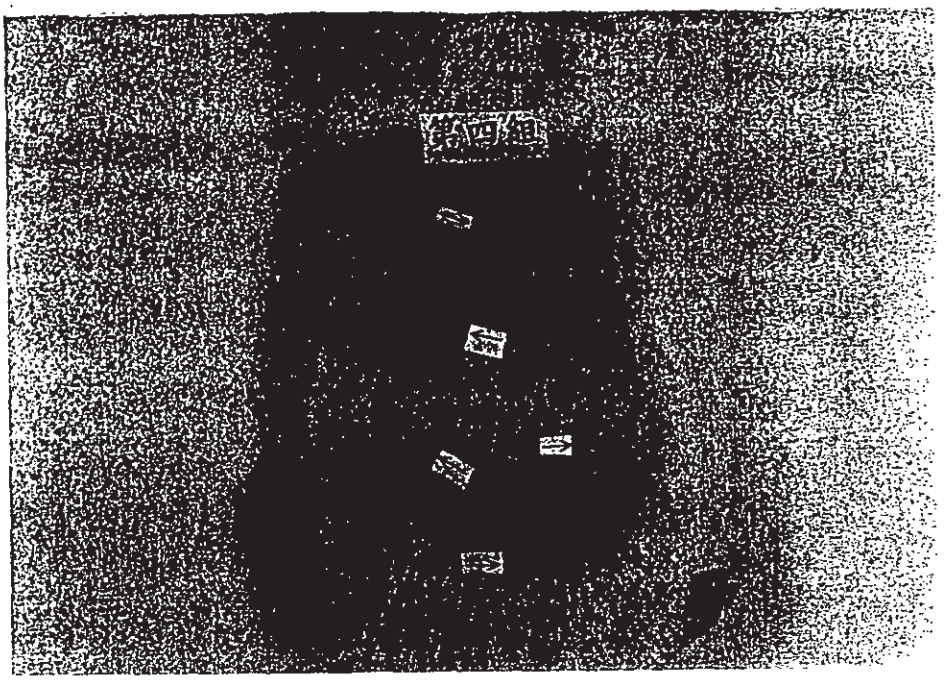


圖 1、各種靈芝膜對於纖維母細胞的毒性分析。(a)表示加靈芝皮(Sacchachitin)；(b)表示加靈芝膜加給丁質，(c)表示加純靈芝膜。縱軸表示各種靈芝膜的濃度，橫軸表示以 MTT 所測得之加入靈芝膜與未加入靈芝膜的細胞之吸光值的比值。

(a)



(b)

圖 2、大白鼠對靈芝皮的過敏反應之觀察。

(a) 表示打入靈芝皮液 24 小時。(b)表示打入靈芝皮液後 7 天。圖中"→"之處爲打入靈芝皮液的地方。

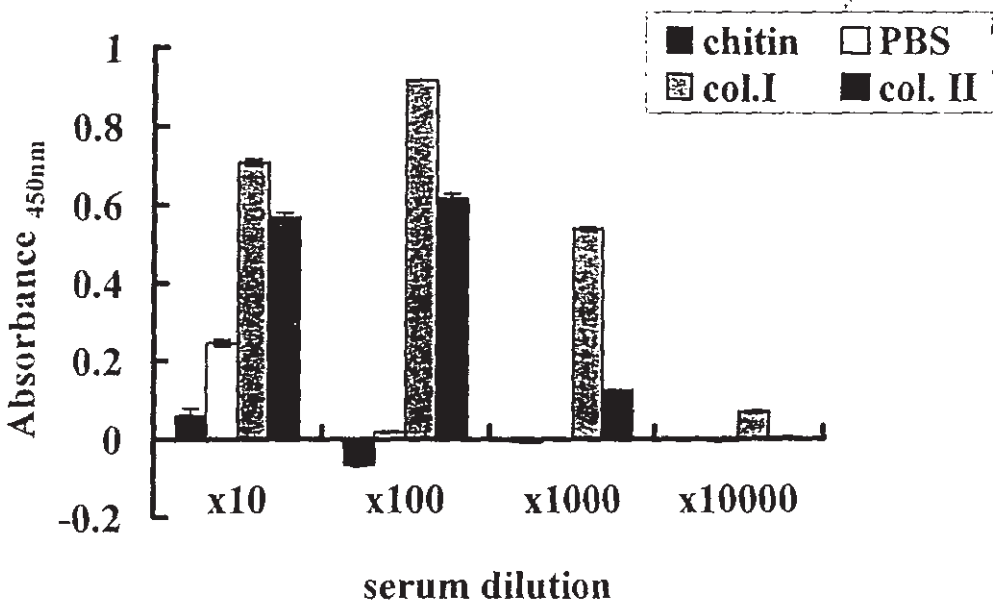
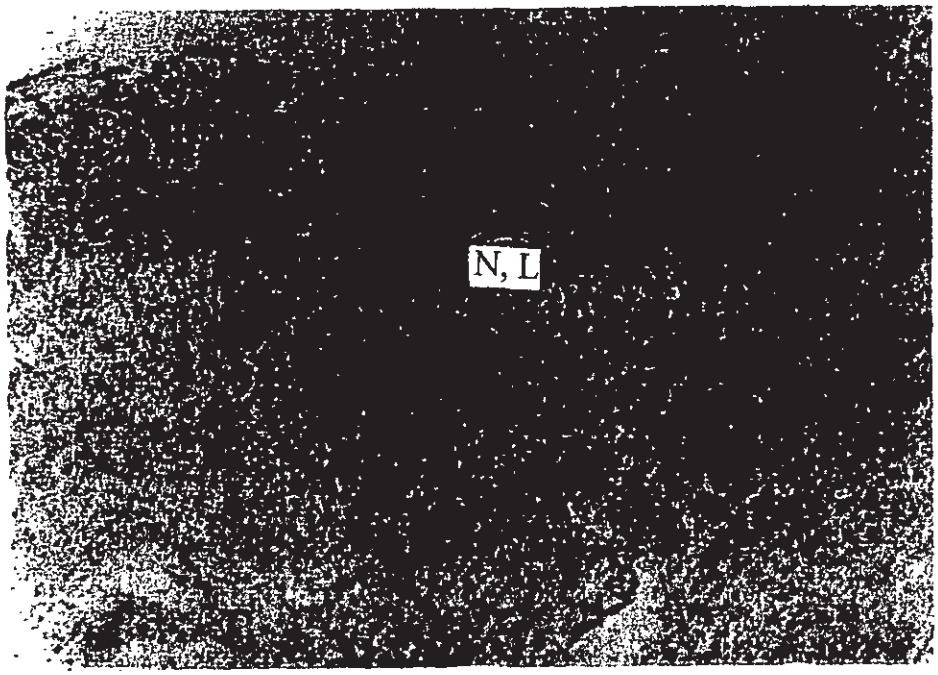


圖 3、靈芝皮對於老鼠所產生的免疫性（immunogenicity）反應之探討。縱軸表示血清以 5% milk 所稀釋的倍數，橫軸表示在 450 nm 波長之下所測得之吸光值。Chitin 表示打入靈芝皮懸浮液後所收集的老鼠血清，PBS 表示打入 PBS 後所收集的老鼠血清，用來當作 negative control，Col. I 及 col. II 分別表示打入 collagen type I 及 collagen type II 後所收集的老鼠血清，當作 positive control。這些所收集的血清皆爲施打抗原三劑後，隔一星期後所收集的血清。

(a)



(b)

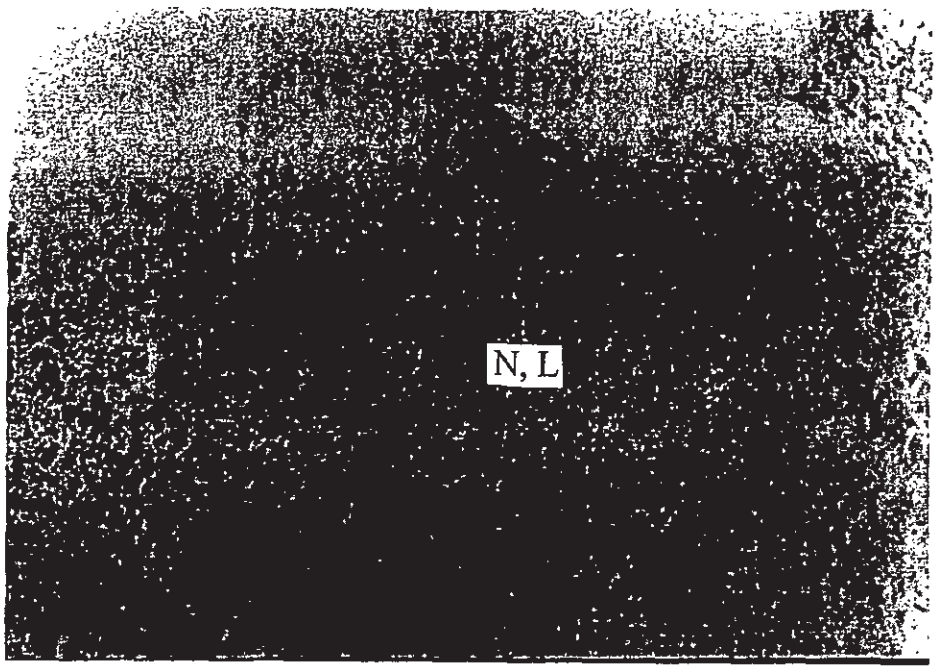
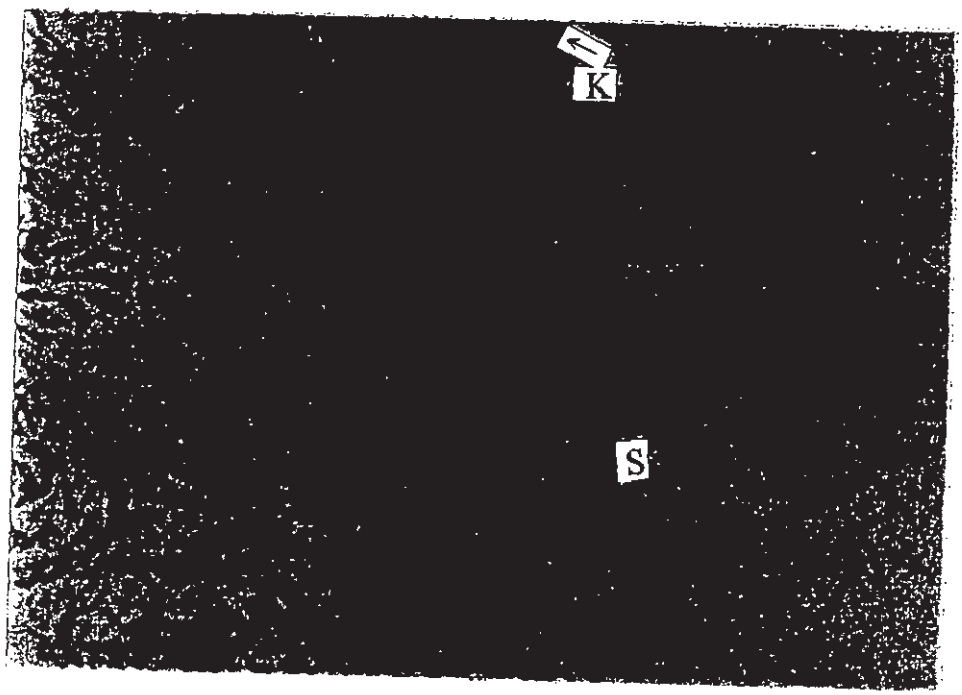


圖 4、大白鼠皮膚第 1 天的傷口之 H-E 染色切片。

(a) 表示覆蓋紗布的傷口。(b) 表示覆蓋靈芝皮的傷口。兩者皆以可見光在 x100 的視野下的觀察。N 表示 neutrophils，L 表示 lymphocyte。

(a)



(b)

圖 5、大白鼠皮膚第 7 天的傷口之 H-E 染色切片。

(a) 表示覆蓋紗布的傷口。(b) 表示覆蓋靈芝皮的傷口。兩者皆以可見光在 x100 的視野下的觀察。S 表示 scar，K 表示角質細胞。