行政院國家科學委員會專題研究計畫　成果報告

中藥梔子素交聯明膠作為神經細胞外間質應用之評估

計畫類別：個別型計畫
計畫編號：NSC92-2320-B-039-040-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：中國醫藥大學學士後中醫系

計畫主持人：呂明進
共同主持人：蔡金川
計畫參與人員：陳悅生

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 10 月 6 日

中藥梔子素交聯明膠作為神經細胞外間質應用之評估

計畫編號：NSC 92-2320-B-039-040 -
執行期限：2003.08.01 至 2004.07.31
主持人：呂明進　中國醫藥大學學士後中醫學系
共同主持人：蔡金川　中國醫藥大學學士後中醫學系
計畫參與人員： 陳悅生　中國醫藥大學中醫所

Abstract

In this report, we describe a novel use of purified genipin, which can be extracted from *Gardenia jasminoides* ELLIS, fixing the gelatin to be an extracellular matrix for peripheral nerve regeneration.

Keywords: Genipin; Gelatin; Peripheral nerve regeneration

中文摘要

本結論報告在說明以矽膠管分別充填明膠與經綠梔子素交聯之明膠，並利用此神經管對截斷 1 cm 的大鼠坐骨神經做一結合，以觀察對大鼠周圍神經再生之影響，並與矽膠管充填膠原蛋白和空管組做一比較。

關鍵詞：明膠；綠梔子素；周邊神經再生

Introduction

The nerve bridge technique, introducing both ends of injured nerve stumps into a tubular chamber, has been shown that it can aid guidance of growing nerve fibers along appropriate paths and can enhance the precision of stump approximation [1-4]. In order to promote the growth of nerves across gaps in the bridging chambers, various stimulatory substances such as laminin, fibronectin, amnion membrane, nerve growth factor, and brain-derived nerve growth factor are loaded into the lumens of the chambers to modify their internal growth microenvironments [5-9]. However, if these substances are in the liquid form as filled in the chambers, they may lose effectiveness within a short period because of diffusion losses through gaps between the stumps and the chamber walls [10]. To overcome this disadvantage, a number of viscous extracellular matrices, such as the collagen, fibrin, hyaluronic acid, Matrigel® and alginate hydrogel have been used to contain the growth-promoting substances in the chamber lumen [11-15]. Nevertheless, if these matrices are not degraded through the proteolysis before adequate axonal regeneration, the remaining gel matrices could impair the regeneration process by physically hindering the migration of neural components, even if the gel contains growth-promoting substances [16]. Therefore, there is still a need for a better understanding for use with extracellular matrices in the reconstruction of injured nerves. Gelatin, which is essentially denatured collagen, has a myriad of uses in the food, pharmaceutical, cosmetic, and industries. It has several

advantages including its unique ability to form gels and its ease of handling. It is also relatively inexpensive and has not shown any antigenicity [17]. However, the gelatin must be cross-linked, if it is used as a stable extracellular matrix without losing its integrity for tissue engineering. The purpose of this study was therefore to investigate if genipin cross-linked gelatin filled in a silicone rubber chamber allowed peripheral nerve regeneration.

Materials and Methods

Before operations, preparation of the collagen and the genipin-fixed gelatin lumen fillings was done.

Collagen lumen filling

A volume of 4.5 ml of chilled Vitrogen® collagen (95% type I and 5% type III bovin dermal collagen in HCl at pH=2.0, Cohesion, Palo Alto, CA) was mixed with 5 ml of 2x phosphate buffered saline solution (0.2 M Na_2HPO_4, 1.3 M NaCl, pH=7.4). The collagen solution was then mixed with 0.5 ml of 0.1 M NaOH. The pH of the solution was neutralized to 7.4±0.2 by the addition of a few drops of 0.1 M HCl or 0.1 M NaOH. Waiting for 10 minutes of cross-linking in an oven at 37 牽 C, we had a final 1.35 mg/ml of collagen solution, which was later filled in the silicone rubber chambers in the collagen group (n=10).

Genipin-fixed gelatin filling

Combination of 3% aqueous gelatin solution fixed with 3% (w/w) of the genipin was used. The genipin-fixed gelatin was first obtained by water-bathing the mixture of 0.6 g of gelatin (75-100 bloom number, Sigma #G6144) with 19.4 ml of distilled water at 80˚C for 1 minute. The gelatin solution was cooled to 40˚C and then fixed with 0.7 ml of genipin (Challenge Bioproducts Co., Taichung, Taiwan) solution for 30 minutes. The genipin-fixed gelatin was then filled in the silicone rubber chambers in the genipin-fixed gelatin group (n=10).

The volume of the chamber lumen was approximately 17.6 μl. Both the fillings were injected in the liquid state through a micropipette into the lumens by passing the tip of the needle inside the silicone rubber chambers. The loading was done as slowly as possible to prevent the formation of air bubbles.

Surgical preparation of animals

Operations were then performed under pentobarbital anesthesia by intraperitoneal injection into the lower left quadrant of the abdomen on male Sprague-Dawley rats, weighing between 300 and 350 gm. Following the skin incision, fascia and muscle groups were separated using blunt dissection, and the right sciatic nerve was severed into proximal and distal segments. The proximal stump was then secured with a single 9-0 nylon suture through the epineurium and the outer wall of the pre-filled silicone rubber chamber (1.47 mm ID, 1.96 mm OD; Helix Medical, Inc., Carpinteria, CA). The distal stump was secured similarly into the other end of the

chamber. Both the proximal and distal stumps were secured to a depth of 1 mm into the chamber, leaving a 10-mm gap between the stumps. In addition to the two experimental groups, animals in the control group (n = 10) received silicone rubber chambers filled with saline only for the evaluation of different extracellular matrices for regenerated nerves. The muscle layer was re-approximated with 4-0 chromic gut sutures, and the skin was closed with 2-0 silk sutures.

Results

After an implantation time of 6 weeks, the implanted silicone implants were retrieved and examined. No inflammation and only brownish fibrous tissue encapsulation was noted covering all over the silicone tube and the parts of the nerve stumps in the tube openings. After trimming the fibrous tissue, the regenerated nerve could be seen through the semitransparent lumen of the tube. It was found that the regenerated nerve, which was surrounded by fluid, occupied a central location within the tube. Overall gross examination of the silicone tubes revealed 80% (8 of 10) and 90% (9 of 10) of nerve formation in the tubes filled with the genipin-fixed gelatin and the collagen gel, respectively, whereas only 30% (3 of 10) in the controls.

Regenerated nerves in the control group displayed a structure with a symmetric and thin epineurium, surrounding a cellular and vascularized endoneurium in which abundant myelinated axons, Schwann cells, and blood vessels were seen (Figure 1). By comparison, regenerates in the groups of the genipin-fixed gelatin and the collagen had a similar structure as those in the control group (Figures 2 and 3). However, residues of fillings among regenerated axons were noted in both the groups of the genipin-fixed gelatin and the collagen (Figure 4). In some of the sections, their endoneurial areas were separated into two parts by the residues of fillings, one had densely packed axons and the other had a loosened structure with isolated axons (Figure 5). The ratio of acellular regions to total area in the denser interior was less than 10%. By comparison, the acellular matrix occupied more than 80% area in the looser exterior region.

Morphometric data of regenerated nerves in different extracellular matrices are listed in Table 1. The mean number of myelinated axons was approximately 5100 (5104±3278) and approximately 8000 (8063±1807) in the genipin-fixed gelatin and the collagen group, respectively. By comparison, the control group had approximately 13400 (13428±1424) myelinated axons, which was significantly larger than both the groups with extracellular matrices ($p=0.01$). However, no significant differences were seen among the three groups for their mean area of myelinated axons, mean endoneurial area, mean total nerve area, and mean number of blood vessels in the regenerates ($p=0.05$)

Discussion and Conclusion

The present study showed that

successful regeneration of nerves across the gap occurred in 80% and 90% of the tubes filled with the genipin-fixed gelatin and the collagen, respectively. These success percentages were significantly higher than that of the controls (30%). Like the collagen, the genipin-fixed gelatin used in this study also provided a suitable microenvironment in the tubes for the growth of regenerating nerves.

In summary, the genipin-fixed gelatin is a suitable extracellular matrix for peripheral nerve regeneration as is the collagen gel. However, the concentration of 3% of the genipin, which was its limit of solubility in the saline, seemed still too high in the matrix. Therefore, it is necessary to further investigate the optimal concentrations for the agents in the matrix, e.g., decreasing the concentration of the genipin, which could provide a better growth environment with fewer barriers for the regenerating nerve.

References

1. Hadlock T, Sundback C, Hunter D, Cheney M, Vacanti JP. 2000. A polymer foam conduit seeded with Schwann cells promotes guided peripheral nerve regeneration. Tissue Eng; 6: 119-27.
2. Den Dunnen WFA, Meek MF, Grijpma DW, Robinson PH, Schakenraad JM. 2000. In vivo and in vitro degration of poly[50/50(85/15L/D)LA/ε-CL], and the implications for the use in nerve reconstruction. J Biomed Mater Res; 51: 575-85.
3. Dahlin LB, Anagnostaki L, Lundborg G. 2001. Tissue response to silicone tubes used to repair human median and ulnar nerves. Scand J Plast Reconstr Hand Surg; 35: 29-34.
4. Sufan W, Suzuki Y, Tanihara M, Ohnishi K, Suzuki K, Endo K, Nishimura Y. 2001. Sciatic nerve regeneration through alginate with tubulation or nontubulation repair in cat. J Neurotrauma; 18: 329-38.
5. Fields RD, Le Beau JM, Longo FM, Ellisman MH. 1989. Nerve regeneration through artificial tubular implants. Prog Neurobiol; 33: 87-134.
6. Satou T, Nishida S, Hiruma S, Tanji K, Takahashi M, Fujita S, Mizuhara Y, Akai F, Hashimoto S. 1986. A morphological study on the effects of collagen gel matrix on regeneration of severed rat sciatic nerve in silicone tubes. Acta Pathol Jpn; 36: 199-208.
7. Woolley AL, Hollowell JP, Rich KM. 1990. Fibronectin-laminin combination enhances peripheral nerve regeneration across long gaps. Otolaryngol Head Neck Surg; 103: 509-18.
8. Bailey SB, Eichler ME, Villadiego A, Rich KM. 1993. The influence of fibronectin and laminin during Schwann cell migration and peripheral nerve regeneration through silicon chambers. J Neurocytol; 22: 176-84.
9. Hollowell JP, Villadiego A, Rich KM. 1990. Sciatic nerve regeneration across gaps within silicone chambers: Long-term effects of NGF and consideration of axonal branching. Exp Neurol; 110: 45-51.
10. Kosaka M. 1990. Enhancement of rat

peripheral nerve regeneration through artery-including silicone tubing. Exp Neurol; 107: 69-77.

11. Chen YS, Hsieh CL, Tsai CC, Chen TH, Cheng WC, Hu CL, Yao CH. 2000. Increased success of peripheral nerve regeneration using silicone rubber chambers filled with an extracellular gel containing collagen, laminin and fibronectin. Biomaterials; 21: 1541-7.
12. Sakiyama-Elbert SE, Hubbell JA. 2000. Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. J Control Release; 69: 149-58.
13. Mosahebi A, Simon M, Wiberg M, Terenghi G. 2001. A novel use of alginate hydrogel as Schwann cell matrix. Tissue Eng; 7: 525-34.
14. Strauch B, Rodriguez DM, Diaz J, Yu HL, Kaplan G, Weinstein DE. 2001. Autologous Schwann cells drive regeneration through a 6-cm autogenous venous nerve conduit. J Reconstr Microsurg; 17: 589-95.
15. Mosahebi A, Fuller P, Wiberg M, Terenghi G. 2002. Effect of allogeneic Schwann cell transplantation on peripheral nerve regeneration. Exp Neurol; 173: 213-23.
16. Valentini RF, Aebischer P, Winn SR, Galletti PM. 1987. Collagen- and laminin-containing gels impede peripheral nerve regeneration through semipermeable nerve guidance channels. Exp Neurol; 98: 350-6.
17. Cenni E, Ciapetti G, Stea S, Corradini A, Carozzi F. 2000. Biocompatibility and performance in vitro of a hemostatic gelatin sponge. J Biomater Sci Polym Ed;11:685-99.

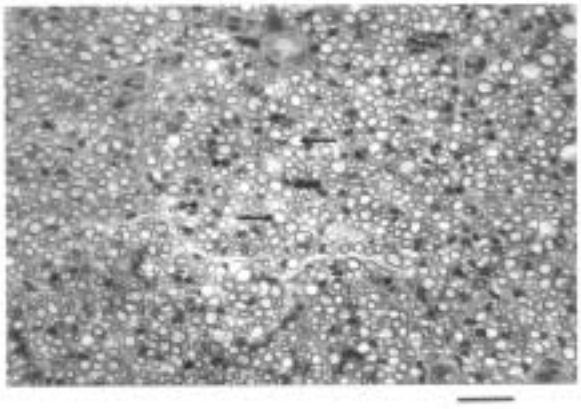

Figure 1

Table 1

Rat No.[a]	No. of Axons (#)	Mean Area of Axons (μm^2)	Endoneurial Area (mm^2)	Total Nerve Area (mm^2)	No. of Blood Vessels (#)
Controls					
5	13809	4.76	0.58	0.66	29
7	11853	3.87	0.39	0.44	56
10	14623	4.28	0.58	0.67	46
Mean	13428	4.30	0.52	0.59	44
SD	1424	0.45	0.11	0.13	14
Collagen					
1	5542	4.40	0.25	0.30	14
2	6577	4.96	0.34	0.43	70
3	10841	5.32	0.22	0.29	86
4	8256	5.23	0.34	0.45	75
5	9852	5.81	0.35	0.42	39
6	7003	5.00	0.32	0.35	46
7	8465	4.69	0.36	0.44	61
9	9644	5.68	0.34	0.40	42
10	6383	4.41	0.38	0.49	45
Mean	8063*	5.06	0.32	0.40	53
SD	1807	0.51	0.05	0.07	22
Gelatin+Genipin					
3	6889	6.30	0.51	0.62	40
4	262	8.69	0.30	0.39	10
5	9808	4.87	0.55	0.63	99
6	8093	6.04	0.46	0.54	43
7	3726	4.41	0.20	0.31	12
8	1201	5.26	0.14	0.18	10
9	5725	6.16	0.36	0.42	33
10	5129	4.62	0.39	0.45	29
Mean	5104*	5.79	0.36	0.44	35
SD	3278	1.38	0.14	0.15	29

[a]Data are not available for rats #1, #2, #3, #4, #6, #8, #9 in the control group, rat #8 in the collagen group, and rats #1 and #2 in the gelatin-genipin group.

*Compared with control group, $p<0.05$

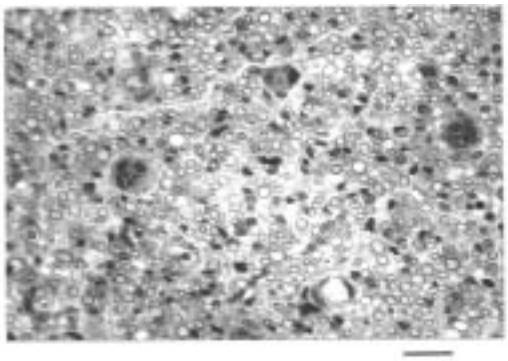

Figure 2

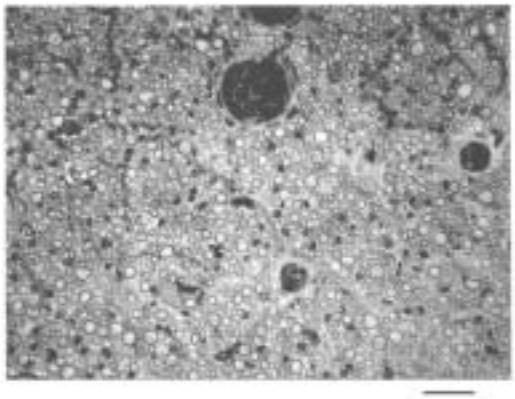

Figure 3

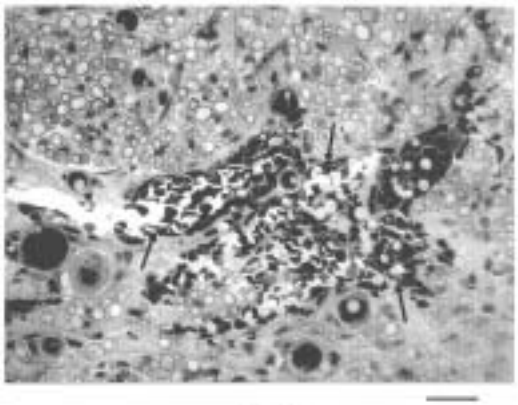

Figure 4

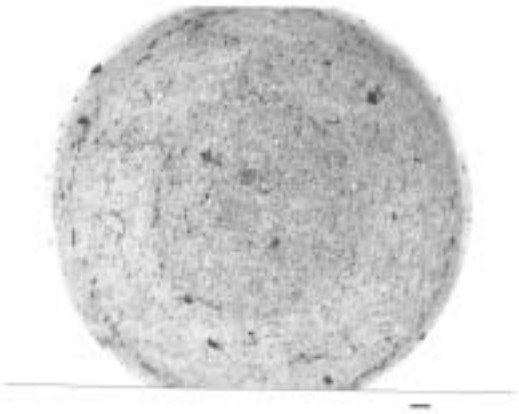

Figure 5A

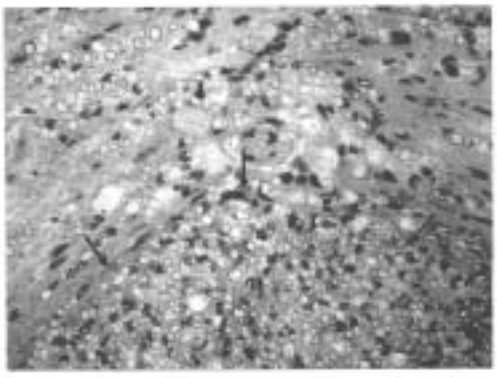

Figure 5B