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論文題目

銀杏葉酯對周邊神經再生之作用評估

Effect of Bilobalide on Peripheral Nerve Regeneration

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1. Introduction

In anastomosis, different neuro-stimulating substances have been used to investigate the possible promotion of the growth of axons in peripheral nerve to cross gaps and grow into the distal segment in a shorter period. These neuro-stimulating substances include nerve growth factor [1], brain-derived neurotrophic factor [2], collagen [3], laminin-containing gel [4], etc. Our group has also successfully demonstrated that the gel mixture of collagen, laminin and fibronectin could offer a suitable growth medium for the regeneration of axons in silicone rubber chambers [5]. Recently, application of combining traditional Chinese medicine and western biomedical science to nerve regeneration is another approach [6,7]. Bilobalide, extraction of the leaves of *Ginkgo biloba*, has been used to treat cerebral ischemia for its capability to improve peripheral arterial occlusion [8]. It has been also reported that the bilobalide has neuroprotective effects and is efficient in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [9]. Although biblobalide has been well characterized from the neuropathic point of view, its effects on nerve regeneration are only little known. Bruno et al. observed the reinnervation of the extensor digitorum longus muscle following the crush of rat sciatic nerve [10]. They found that the

rearrangement of regenerated innervation occurred more rapidly in bilobalide-treated animals. Similarly, Zhang et al. reported that oral administration of biblobalide-containing solution could promote the regeneration of crushed rat sciatic nerve [11]. However, both the studies with the nerve regeneration model have only used a short nerve gap. The inherent regenerative capacity of the peripheral system in animals is so efficient over shorter gaps that the benefits of the bilobalide may not be fully revealed. In this study, we therefore used a silicone rubber chamber filled with a mixture of bilobalide and collagen for establishing a nerve bridge across a 15-mm gap for rat sciatic nerves. Histological and electrophysiological techniques were used to evaluate the functional recovery of the nerve.

2. Materials and Methods

Surgical preparation of animals

Fifty adult Sprague-Dawley rats underwent placement of silicone chambers. The animals were anesthetized with an inhalational anesthetic technique (AErrane®, Baxter, USA). 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 silicone rubber chamber (1.47 mm ID, 1.96 mm OD; Helix Medical, Inc., Carpinteria, CA). Animals were divided into 5 groups outlined in Table 1.

Table 1: Experimental groups

Group	Variable	Rational
Group A (n = 10)	Collagen + Saline	Control
Group B (n = 10)	Collagen + 50 μM bilobalide	Low dose of bilobalide
Group C (n = 10)	Collagen + 100 μM bilobalide	Medium-low dose of bilobalide
Group D (n = 10)	Collagen + 200 μM bilobalide	Medium-high dose of bilobalide
Group E (n = 10)	Collagen + 400 μM bilobalide	High dose of bilobalide

In group A (n = 10), the chambers were filled with a mixture at a concentration of 2.4 mg/ml collagen (Vitrogen®, Cohesion, Palo Alto, CA) and normal saline in a 1:1 volumetric addition. In group B (n = 10), a mixture of the Vitrogen® collagen (2.4 mg/ml) and 50 µM bilobalide (Sigma) in a 1:1 volumetric addition was filled in the chambers. Similarly, chambers in groups C (n = 10), D (n = 10), and E (n = 10) were filled with a mixture of the Vitrogen® collagen (2.4 mg/ml) and 100, 200, and 400 µM bilobalide (Sigma) in a 1:1 volumetric addition, respectively.

The volume of the chamber lumen was approximately 25.5 µl. These fillings, which were in the liquid state, were injected through a pre-cooled micropipette into the lumens by passing the tip of the needle inside the silicone rubber chambers and the loading was done as slowly as possible to prevent the formation of air bubbles. The mixture polymerized into a gel at 37°C of the animal body temperature. The distal stump was then secured 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 15-mm gap between the stumps. The muscle layer was re-approximated with 4-0 chromic gut sutures, and the skin was closed with 2-0 silk sutures. All animals were housed in temperature (22°C) and humidity (45%) controlled rooms with 12-hour light cycles, and

they had access to food and water *ad libitum*.

All chambers remained in place for 8 weeks, at which time the nerves were re-exposed and the chambers examined for the presence of regenerated nerve across the 15-mm gap. Those animals with apparent nerve regeneration were injected with horseradish peroxidase (HRP) in the tibial nerve. The tibial nerve was crushed for 1 minute, and was injected by micropipette of 5 µl of 20% HRP. The injection was applied as slowly as possible to prevent spillage of the HRP. The wounds were closed for another 48 hours before the animals were sacrificed.

Electrophysiological techniques

All the animals with apparent nerve regeneration were re-anaesthetized and the sciatic nerve exposed. The sciatic nerve was stimulated with supramaximal stimulus intensity through a pair of needle electrodes placed directly on the sciatic nerve trunk, 5-mm proximal to the transection site. Amplitude and latency of the evoked muscle action potentials were recorded from gastrocnemius muscles with micro-needle electrodes linked to a computer system (Neuropack, Nihon Kohden Co., Japan). The latency was measured from stimulus to the takeoff of the first negative deflection and the amplitude from the baseline to the maximal negative peak.

Histological techniques

Immediately after the recording of muscle action potential, the HRP-labeled animals were transcardially perfused with 200 ml of 9% saline, followed by 500 ml of 2% glutaraldehyde/1.25% paraformaldehyde in 0.1 M PBS, pH 7.4. The gastrocnemius muscle at the operated and the non-operated sites, the regenerated sciatic nerve, and the lumbar portion of the spinal cord at levels 4, 5, 6 were removed. Before processing, the gastrocnemius muscle was weighted. The ratio of the gastrocnemius muscle weight between the operated and the non-operated sites was used as an indirect evidence of nerve regeneration. The muscle specimens were then dehydrated, embedded in paraffin, cut and stained with hematoxylin and eosin. Sciatic nerve sections were taken from the middle regions of the regenerated nerve in the chamber. After the fixation, the nerve tissue was post-fixed in 0.5% osmium tetroxide, dehydrated, and embedded in spurs. The tissue was then cut to 5-μm thickness by using a microtome with a dry glass knife, stained with toluidine blue. The spinal cord was placed in 0.2 M phosphate buffer with 30% sucrose at 4°C for overnight, embedded in frozen resin (Cryomatrix^{TM}, Shandon Inc., Pittsburgh, PA), sectioned longitudinally at 40 μm, and collected in 0.1 M of phosphate buffer. The tissue was then processed with tetramethyl benzidine, serially

mounted on chrome-alum slides, counterstained with neutral red, and dehydrated. All tissue samples were observed under a light microscope (Olympus IX70, Olympus Optical Co., Ltd., Japan). An image analyzer system (Image-Pro Lite, Media Cybernetics, USA) coupled to the microscope then counted the blood vessels and calculated the cross-sectional area of each nerve section at magnifications between 40x and 400x. As counting the axons, at least 30 to 50 percent of the nerve section area randomly selected from each nerve specimen at a magnification of 400x was observed. The axon counts were extrapolated by using the area algorithm to estimate the total number of axons for each nerve. All data are expressed as mean ± standard deviation. Statistical comparisons between groups were made by the Student's t-test.

3. Results

Since we used a long size defect to evaluate the nerve regeneration, a small number of replications used for comparisons were expected. Though the small replication number may not reflect practical correlations among cable sections, however, in conjunction with noting the successful rates of nerve regeneration and how the morphometric data clustered in the grouping comparisons, meaningful differences could be identified.

Gross examination of the silicone rubber chambers at 8 weeks revealed high rates of successful regeneration in the groups of bilobalide at 100 and 200 μM, with 50% (5 of 10) and 40% (4 of 10) of the animals exhibiting a regenerated nerve cable across the 15-mm gap, respectively. In comparison, only 10% (1 of 10) of the animals in the group of bilobalide at 400 μM and in the controls showed such regenerated nerve cables. As for the animals treated with the bilobalide at 50 μM, no regenerated nerve cables were seen (Figure 1).

Figures 2a-2d show representative cross sections of regenerated nerve specimen. Regenerated nerves selected from all the tube-grafted groups displayed a similar

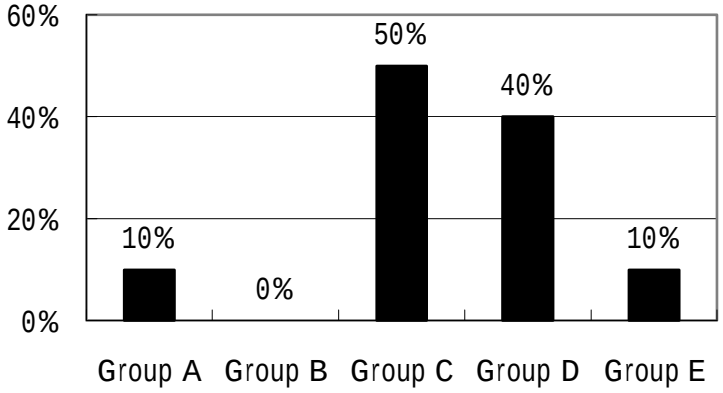

Figure 1: Success rates of regenerated nerves across the 15 mm gaps

ultrastructural organization. The epineurial and perineurial regions of the regenerated nerves consisted mainly of a collagenous connective tissue matrix in which circumferential cells resembling perineurial cells and fibroblasts were seen. In addition, the nerve fibers were loosely distributed in the endoneurium. Nuclei of Schwann cells were interspersed among these nerve fibers. Axons in the endoneurium were easily defined by their surrounding myelin sheaths stained dark blue by the toluidine blue. Morphometric data revealed that the bilobalide at 200 μM significantly increased the number of myelinated axons that grew into the distal end of the chamber by approximately two-fold as compared with the other groups (Table 2). In addition, blood vessels were numerous in the epineurium as well as in the endoneurial areas of the nerve. Motoneurons of the lumbar spinal cord were retrogradely labeled by applying HRP distal to the regenerated nerve cable, revealing the successful neuronal

Figure 2: Light micrographs of representative nerve cross sections from group A (panel a, collagen + saline), group C (panel b, collagen + 100 µM bilobalide), group D (panel c, collagen + 200 µM bilobalide), and group E (panel d, collagen + 400 µM bilobalide). Scale bars = 30 µm (same magnification for a, b, c and d)

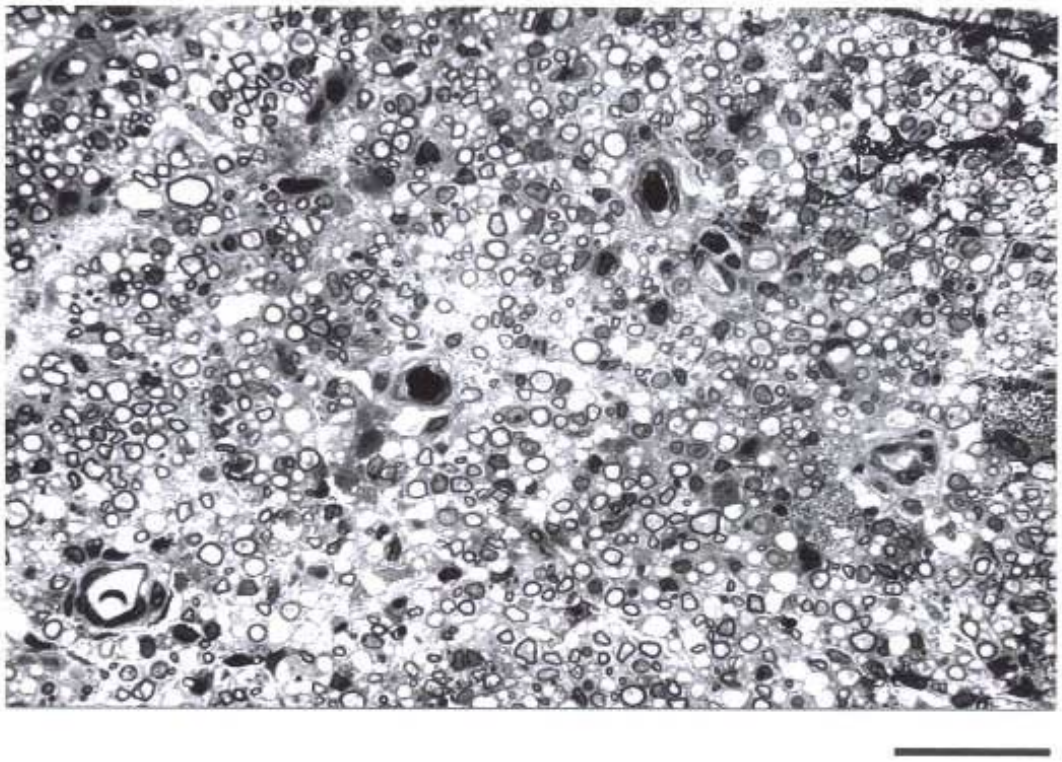

Figure 2a

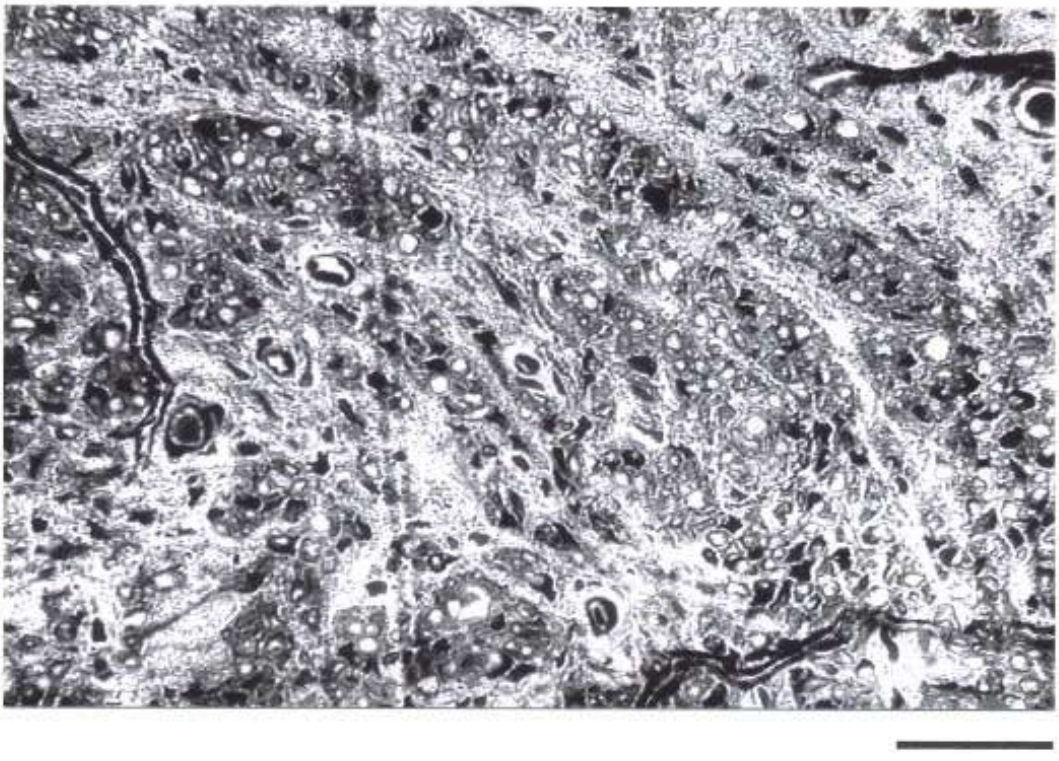

Figure 2b

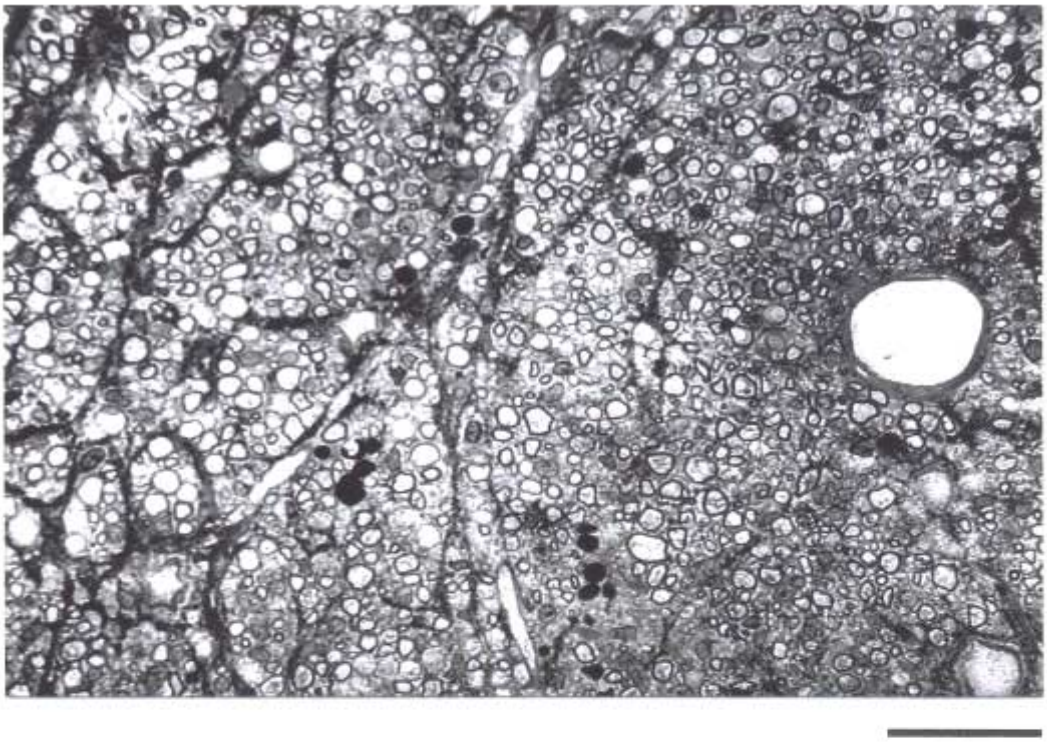

Figure 2c

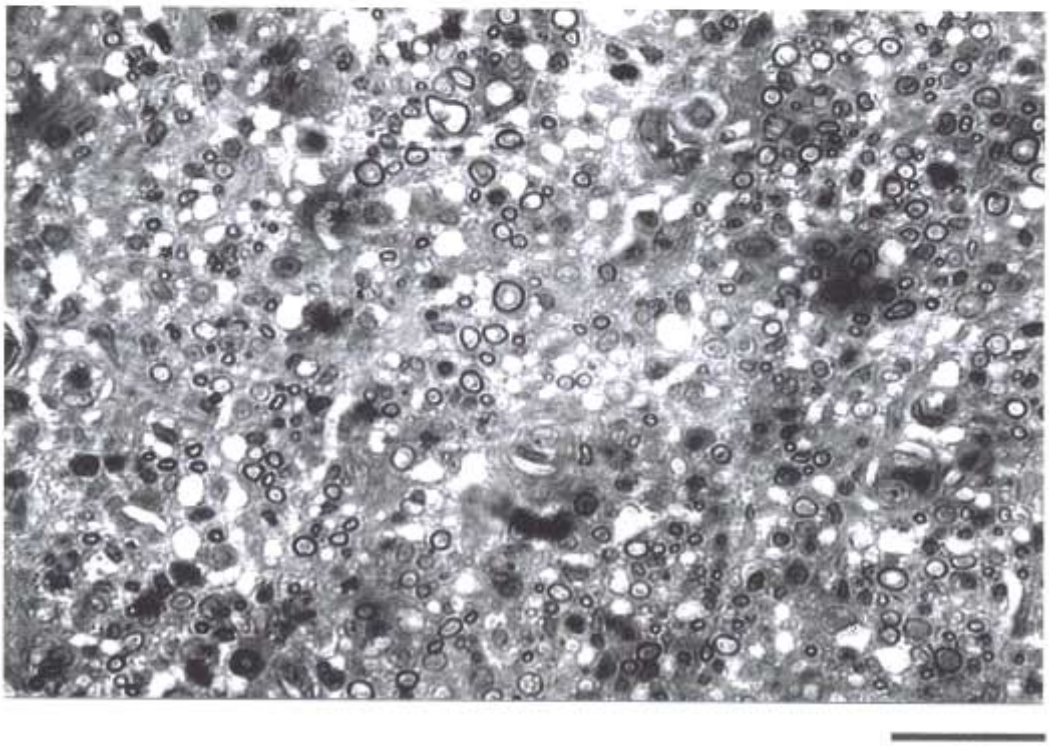

Figure 2d

Table 2: Morphometric data from the regenerated nerves in the chambers

Rat No.	No. of Axons (#)	Mean Area of Axons (μm^2)	Endoneurial Area (mm^2)	Total Nerve Area (mm^2)	No. of Blood Vessels (#)	Muscle Weight Ratio	Latency (ms)	Base to Peak Amplitude (mv)
Group A								
1	2485	8.93	0.80	0.89	72	0.41	1.50	1.00
Group B	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Group C								
4	0	0.00	0.01	0.13	0	0.40	0.00	0.00
6	175	5.55	0.05	0.05	1	0.33	1.42	2.93
7	4009	5.74	0.35	0.41	69	0.63	1.42	1.67
8	5662	10.23	0.59	0.69	121	0.53	0.96	4.09
10	1708	6.52	0.20	0.25	100	0.33	1.36	1.76
Mean	2311	5.61	0.24	0.31	58	0.44	1.03	2.09
SD	2469	3.66	0.24	0.25	56	0.13	0.61	1.53
Group D								
1	3181	9.10	0.59	0.71	107	0.43	1.56	1.42
2	5085	8.53	0.47	0.55	31	0.60	1.24	1.98
3	2406	8.95	0.48	0.54	55	0.61	1.13	0.93
6	5702	4.54	0.55	0.64	112	0.46	1.16	1.96
Mean	4094	7.78	0.52	0.61	76	0.53	1.27	1.57
SD	1555	2.17	0.06	0.08	40	0.09	0.20	0.50
Group E								
1	1975	7.37	0.40	0.51	56	0.41	1.24	2.24

connectivity. The colorful contrast between the dark granules of reaction-product and white nucleus made it easy to detect the labeled neurons (Figure 3).

Light microscopy of the gastrocnemius muscle is shown in Figures 4a-4b. As compared to normal muscle fibers, those from injured sites of the animals in all the groups became smaller and rounder and displayed a looser structure with dramatically increased nuclei. Ratios of the muscle weight between the operated and the non-operated sites were small in all the groups, indicating that the atrophy of the muscle was still serious even after 8 weeks of recovery. Large variations were seen in the maximum amplitude and latency in all the groups, which could also result from the un-recovered muscle tissue.

Figure 3: Light micrograph of an HRP-stained motor neuron. Dark granules of reaction-product and white nucleus are easily discerned. Scale bar = 30 µm

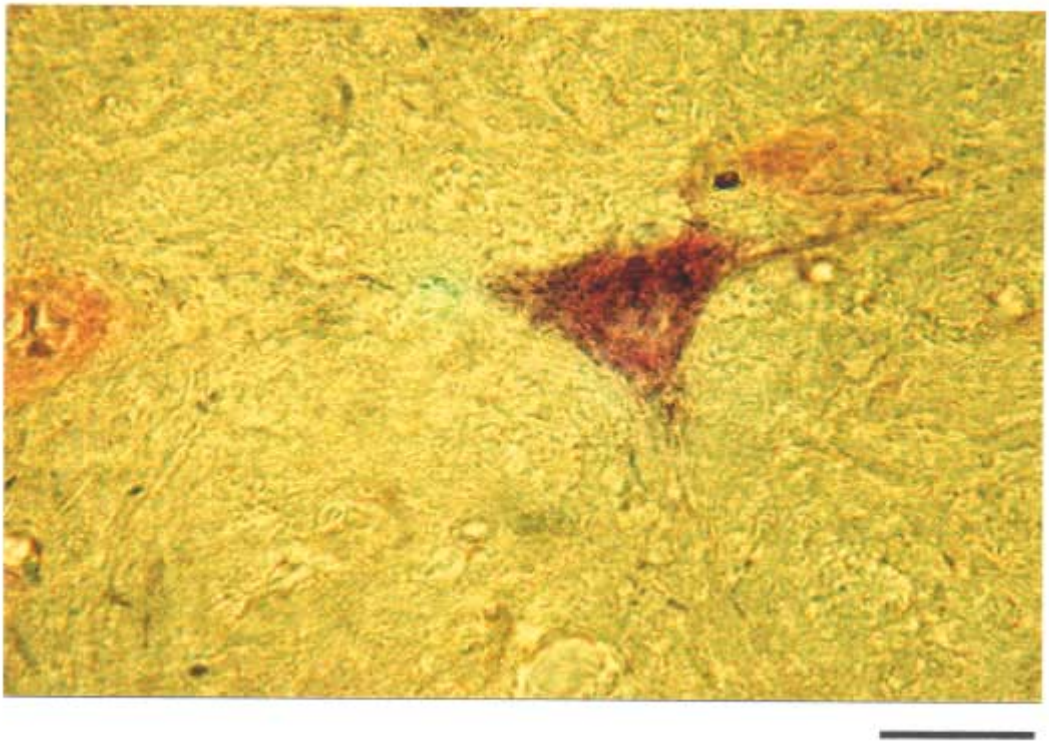

Figure 3

Figure 4: Normal rat gastrocnemius muscle (a). Cross section of the muscle bundle shows closely packed fibers. The nuclei appear at the periphery. Evident muscle atrophy is noted in all the tube-grafted groups (b). The nuclei become dramatically increased. Increased fibrosis and fatty infiltration are also seen. Scale bars = 100 μm (same magnification for a and b)

Figure 4a

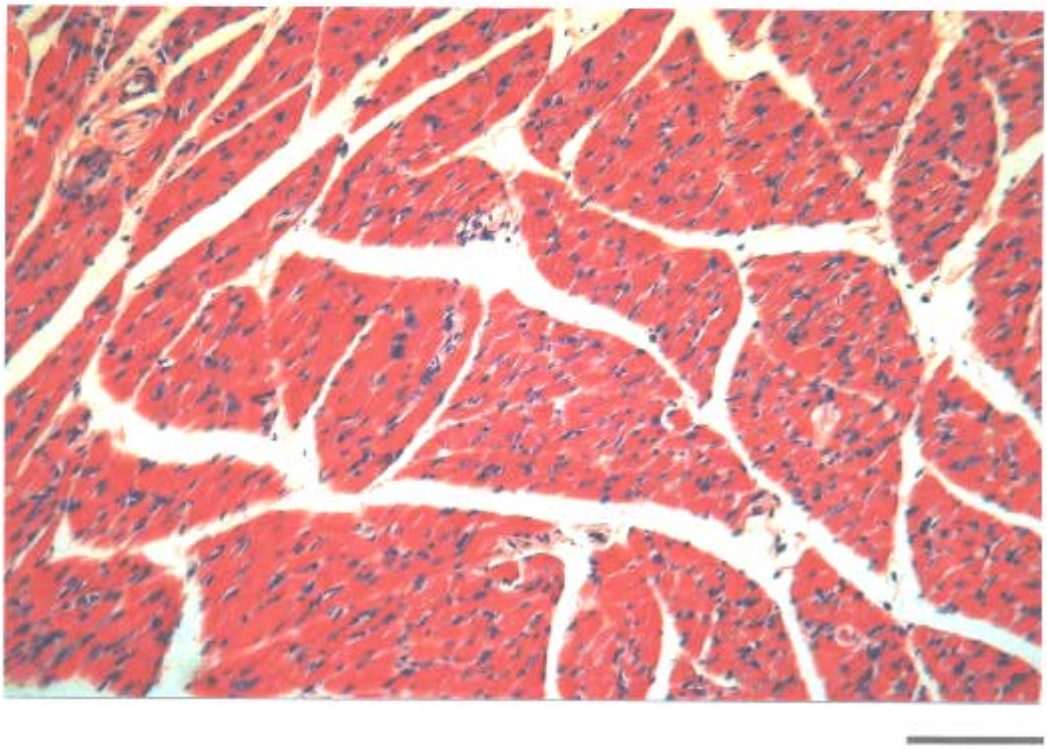

Figure 4b

4. Discussion

A nerve bridge technique is the introduction of both ends of the injured nerve stumps into a tubular chamber, which can aid guidance of growing nerve fibers along appropriate paths and can enhance the precision of stump approximation. But, what bases should be used to define a so-called “successful nerve regeneration” within the guidance chamber? In our opinion, the percentage of regenerated nerve successfully crossing a long length of the gap and the maturity of nerve microstructure should be the most important indications to assess regenerated nerves. Most studies suggest that the critical nerve defect in rats is 10 mm [12,13]. Therefore, a nerve gap length longer than 10 mm should be used to fully reveal the benefits of modification in the bridging chamber. In the present study, we therefore filled a mixture of collagen and bilobalide in silicone rubber chambers to repaired dissected rat sciatic nerves with 15 mm apart. Collagen is not only a commonly used neurite-promoting factor, but also a good extracellulat matrix [14]. After cross-linking, the collagen becomes viscous and is a suitable encapsulation material. As for the bilobalide, it is a terpene lactone in the *Ginkgo biloba* [15], which has long been used in Asia as a drug to invigorate weak or senior people. In modern pharmacology, bilobalide has been shown to exert

neuroprotective and antiapoptotic effects *in vitro* [16]. It has been also successfully used to protect brain tissue against hypoxic damage in mice and rats [17]. In addition, it is efficient in human patients with dementia [18]. From those aforementioned studies, it shows that bilobalide may exert positive effects on the nervous system. In this study, we also found that administration of an appropriate dosage of bilobalide could significantly enhance the formation of a nerve cable across the wide nerve gap in the silicone rubber chamber. Fifty and 40% of the animals in the medium-low and the medium-high dose of bilobalide-treated groups had cables that grew across the gap, respectively. In comparison, only 10% of the animals in the control group exhibited such bridging cables. It is also important to note that bilobalide not only could increase the rate of successful regeneration, but also enhance axonal growth within the nerve cable, especially in the group of medium-high dose of bilobalide. In this group, approximately two-fold axons (4094±1555) successfully grew across the 15-mm gap as compared to the controls (2485). Several possible mechanisms related to the growth-promoting effects mediated by bilobalide have been raised. It has been suggested that bilobalide's neuroprotection capability could be through its antioxidant properties and effects on cerebral glucose and lipid metabolism as well as on neurotransmitters [19]. This neuroprotection effect exerted by bilobalide could also simultaneously attenuate the degeneration and boost the regeneration of the injured nerve. In addition, Bruno et al.

found that the rearrangement of regenerated innervation occurred more rapidly in bilobalide-treated animals [10]. They believed that the poor water solubility of the bilobalide and its capacity to form stable complexes with phospholipids were factors to influence the time course of multiple innervation. Another possible explanation is that bilobalide could decrease the concentration of Ca^{2+} in the Schwann cells, preventing the occurrence of neurochemical damages [11].

Though the capacity of bilobalide to potentiate neurite outgrowth in developing nerves is beyond doubt, it still needs to be very carefully as applying it clinically. In this study, we found that the growth-promoting capability of bilobalide seemed to be suppressed as its concentration was boosted to 400 μM. Only 10% of the animals treated with the high dose of bilobalide had the regenerated cables. We believed that an excessive dosage of bilobalide could provoke adverse responses to the recovery of regenerated nerves. Our finding is similar to that of Gallo et al. [20]. They found that the responses of cultured chick dorsal root ganglion neuronal growth cone to NGF-coated polystyrene bead were prevented as elevating the background NGF concentration. They believed the two receptors on neuron, trkA and $p75^{NTR}$, were saturated by the increased extracellular growth-promoting substances, thereby blocking the responses. Their findings were supported by Boyd and Gordon indicating that

axonal regeneration could be inhibited by the administration of high doses of brain-derived neurotrophic factor by functional blockade of $p75^{NTR}$ receptors [2]. Mohiuddin et al. and Hirata et al. also reported that excessive nerve growth-promoting substances could suppress the axotomy-induced elevation of growth-associated protein 43 (GAP-43), resulting in inappropriate reestablishment of injured nerve [21,22]. Their findings may explain why the rate of successful regeneration in the group of high dose of bilobalide became so low.

We also found that the electrophysiology, which is an indication of the degree of maturation of regenerated nerve fibers [23], did not support the histological findings in the present study. This result was not surprising since the atrophy of the muscle was still serious after 8 weeks of recovery, implying that that the muscle was immature even if muscle fibers had been reinnervated.

In conclusion, the current work reports the first use of the mixture of bilobalide and collagen in silicone rubber chamber and shows that this combination significantly promoted peripheral nerve regeneration across a wide gap. Although the results are encouraging, the inhibitory actions of high dose bilobalide on axonal regeneration implicated the importance of using a proper dosage of this factor in clinical treatment.

Otherwise, an overdosed bilobalide could provoke quite different and unexpected responses, resulting in detriment to the recovery of regenerated nerves.

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銀杏葉酯對周邊神經再生之作用評估

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本實驗利用內填不同濃度 (0, 50, 100, 200, 400 μM) 的銀杏葉酯之矽膠神經管來修補大鼠截斷 15 mm 的坐骨神經，膠原蛋白亦添加於管內以防止銀杏葉酯的滲漏，並利用病理學及電生理學來評估再生神經功能恢復的情形。經 8 星期的實驗期後，相較於管內不加填任何藥物的對照組 (10%, 2485)，加填銀杏葉酯的實驗組，尤其在銀杏葉酯濃度為 200 μM 時，神經再生的成功率 (40%) 及再生軸突的數目均明顯的增加 (4094±1555)。但是當管內加添高劑量銀杏葉酯時，反而造成神經再生成功率的大幅下降 (10%)。此實驗說明了銀杏葉酯對再生神經同時有促進及抑制作用，是否使用洽當劑量的銀杏葉酯則是決定神經再生成功與否的關鍵。

關鍵字：銀杏葉酯、矽膠神經管、周邊神經再生

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