Effectivity of Handmade Tubular Lyophilized Amnion Membrane as A Nerve Conduit in Repair of Peripheral Nerve Injury with 5 mm Gap in Rats

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ABSTRACT

Introduction. Peripheral nerve injury with 5–30 mm gap which is caused by direct injury cases (87%) or iatrogenic (12%) become a special concern because it may cause a serious disability in the future. Therefore, we need many kind of nerve repair methods without adding morbidity to the patient. One of the methods is entubulation method, by using natural or synthetic material.

Materials & Methods. This is an animal experimental research by using post-test only control group design in Pharmacology Laboratory Faculty of Medicine Universitas Padjadjaran Bandung in May 2012. In this study, we used 14 Wistar rats divided into 2 groups. After creating gap on sciatic nerve, nerve conduit is installed on treatment group by using handmade tubular lyophilized amnion membrane. Nerve conduit is not installed on control group. After 21 observation days, conduction test and histopathology examination were done. Data was analyzed using non-parametric statistical analysis Sign test.

Results. Result showed significant difference between two groups; the conduction test=0.016 ($p<0.05$), nerve growth to distal gap=0.063 ($p<0.05$), no radial direction of nerve growth=0.031 ($p<0.05$). Reaction of inflammation was minimal and there was no difference between two groups.

Conclusions. Handmade tubular lyophilized amnion membrane is effective as nerve conduit in repair of peripheral nerve injury with 5 mm gap.

Key words: Handmade tubular lyophilized amnion membrane, nerve conduit, peripheral nerve injury

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Efektivitas Membran Amnion Liofilisasi *(Handmade Tubular)* sebagai *Nerve Conduit* di Perbaikan Cedera Saraf Perifer Tikus dengan Celah 5 mm

ABSTRAK

Pendahuluan. Cedera saraf perifer dengan *gap* sekitar 5–30 mm baik akibat cedera langsung (87%) maupun iatrogenik (12%) mendapat perhatian khusus karena dapat mengakibatkan kecacatan di kemudian hari. Untuk itu dibutuhkan metode perbaikan saraf dengan tanpa menambah morbiditas pada pasien, salah satunya dengan metode entubulasi, berbahan alamiah atau sintetik.

Bahan dan cara kerja. Penelitian berupa eksperimental hewan coba di Laboratorium Farmakologi Fakultas Kedokteran Universitas Padjadjaran Bandung pada Mei 2012. Sampel tikus Wistar dewasa dengan jumlah 14 dibagi menjadi 2 kelompok. Setelah dibuat gap pada saraf ischiadikus, kelompok perlakuan dilakukan pemasangan *nerve conduit* dengan bahan membran amnion liofilisasi yang telah dibuat secara manual sebelumnya (*handmade tubular*). Kelompok kontrol tanpa pemasangan *nerve conduit*. Setelah observasi selama 21 hari, dilakukan uji konduksi dan pemeriksaan histopatologi. Data diolah dengan analisis statistik non-parametrik *Sign test*. Semua hewan coba selamat tanpa ada yang mengalami komplikasi pascaoperasi.

Hasil. Dari penelitian didapatkan perbedaan yang signifikan antara kelompok perlakuan dengan kontrol, uji konduksi sebesar 0,016 ($p<0,05$), pertumbuhan saraf hingga *distal gap* sebesar 0,063 ($p<0,05$), arah pertumbuhan saraf yang tidak radier sebesar 0,031 ($p<0,05$). Pada reaksi peradangan tampak minimal dan tidak terdapat perbedaan antara kedua kelompok.

Simpulan. Membran amnion liofilisasi efektif untuk digunakan sebagai *nerve conduit* dalam perbaikan cedera saraf perifer disertai *gap* 5 mm.

Kata kunci: cedera saraf perifer, membran amnion liofilisasi, *nerve conduit*

Introduction

Peripheral nerve injury as a relative complication which caused by direct trauma (87%) or iatrogenic, such as in tumor eradication surgery (12%), becomes a special concern because it may cause a serious disability in the future.[1-5] The predilection for this kind injury is 81% in upper extremity and 11% in lower extremity.[6]

In mild nerve injury, recovery come spontaneously but in severe one (neurotemesis) with gaps there will be fibrous tissue across the gaps which can inhibit axonal regeneration from proximal to distal stump.[1] To avoid this problem, bridge operation is therefore preferred to the gaps with purpose to facilitate axonal regeneration to distal stump.[1,7]

If regenerating units do not reach the endoneural environment of the distal stump (for instance, if they are blocked by scar tissue), then they will form neuromas that result in a loss of potential nerve function.[2]

Various surgical options and their outcomes for the management of peripheral nerve injury gaps. If the distance gap is ≥ 5mm, it will cause excessive tension that results in scar and poor vascularization at the repair site.[6] For repair of gaps between 5-30 mm, the gold standard for bridging the proximal and distal stumps is still the nerve autograft.[1-8] But if the gaps longer than 30 mm or for those very proximal ones in which the spinal nerve root has been or are likely avulsed from the spinal cord, the use of nerve transfers has emerged.[2,7]

Using nerve autograft to repair the gaps has several shortcomings including long surgery time, donor site morbidity with neuroma and scar tissue, the limited number and diameter of donor nerves inadequate lead to less optimal of nerve repair.[2,3,8-11] Various results of using nerve autograft, and some studies reported unsatisfying results.[2]

This problem has led to the development of new techniques to bridge the nerve gap. One of them is utilizing a tubular nerve guidance channel or nerve conduit, such natural or synthetic guidance channels are being developed as alternatives to autografts. This

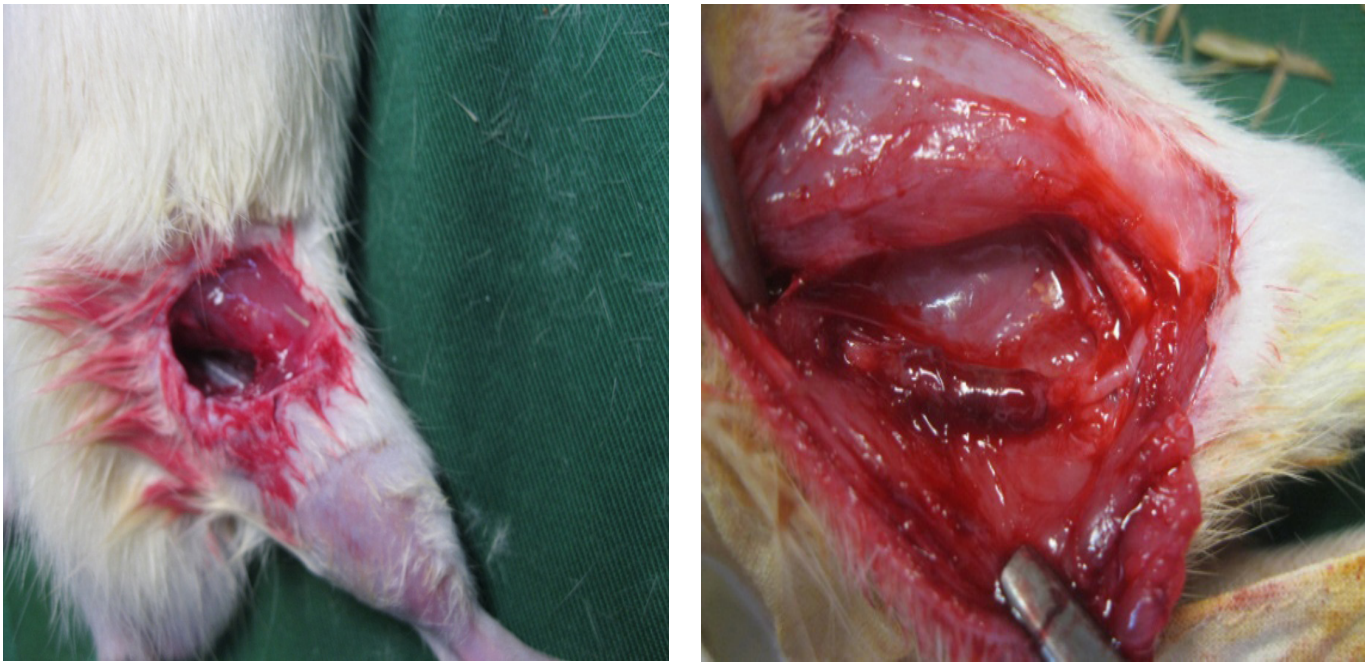

Figure 1. Nerve conduit applied to bridge the nerve gap in intervention group (left) and result after 21 days (right).

technology of "entubulation repair" has several theoretical advantages over nerve autograft, as these nerve conduits help direct axonal sprouts from the proximal stump to the distal nerve stump. They also provide a channel for diffusion of neurotropic and neurotrophic factors secreted by the Schwann cells of the injured distal nerve stump and minimize infiltration of fibrous tissue.

There are some special characterization for the materials to gain successful regeneration of the nerve: biocompatible to the surrounding nerve tissue; minimal inflammation reaction; stimulate axonal regeneration; and biodegradable after the nerve has healed.[7,11] In addition to their biochemical properties, nerve conduit must also possess certain mechanical properties, like easily produced, feasible, semi-flexible and easy to manipulate in surgery.[11]

Many kind of materials as tubular nerve guidance channel has been reported, such as natural one like collagen, muscle, basal lamina, vein graft, artery, or synthetic one biodegradable like polyglycolic acid, polyactic acid, polylacticcoglycolide acid, poly-DL-lactide-co-glicolide, polycaprolactone, polylactide-caprolactone, poly-3-hydroxybutyrate, laminin, poliuretan, and polyphosphazene or non-biodegradable like silicon.

Biological nerve guides or nerve conduit has several shortcomings such as tissue reaction, fibrous tissue formation, scar tissue infiltration and leak of mechanical precision.[12]

One drawback of biodegradable synthetic nerve conduit is the release of toxic degradation products. These products will stimulate macrophage invasion, fibrous tissue and uncoordinated axonal growth.[2,8] The available conduits in the market such as Neuragen, Neuromatrix, Neuroflex, Neurotube and Neurolac have been recognized by US Food and Drug Administration and Conformit Europe with the price ranged between €350 to 1 200.[1,8,12,13] This price range creates a significant financial burden to patients if they are to be applied in our country. Non-biodegradable nerve conduits such as silicon has some disadvantages as they cause inflammatory reaction and fibrous tissue formation, tend to create chronic compression of the nerve, and also the need for second surgery for removal after the nerve has healed.[1]

To overcome these problems, an alternative method using human amniotic membrane as a nerve conduit to bridge the gap of the nerve injury have been proposed.[14,15] Amniotic membrane has biocompatible characterization to the surround nerve tissue and could reduce the risk of fibrous formation by reducing tumor growth factor-β regulation and stimulate new vascularization.[11,15] Human amniotic membrane matrix could also stimulate neuron regeneration in vitro and in vivo.[11]

In previous studies, nerve conduit made using fabricated human amniotic membrane, known as an amnion tube or amnion matrix tube which are applied to a sciatic nerve with gap in rats and compared the result

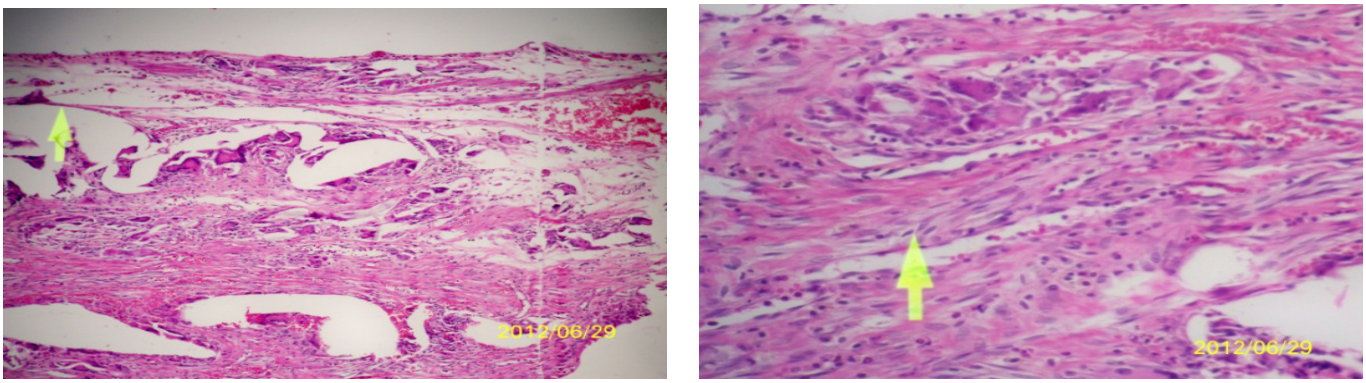

Figure 2. Histopathological of intervention group at distal gap at 40x(left) and 400x(right). Arrow Sign shows nerve cell

to nerve conduit with materials such as silicon and nerve autograft by histopathology. Results of that study have found that the amnion tube or amnion matrix tube is effective to be used as a nerve conduit in nerve injury with gap.[11,15]

Materials and methods

This was an animal-based experimental study using 2.5 to 3 months old Wistar Rats with body weights averaging at 300-350 gr. This study was performed from May to June 2012.

The inclusion criterion was rats with good function of sciatic nerve, while the exclusion criterion is if there are wounds or skin infection around the planned incision area. Drop-out criterion are when the animals experienced premature termination and surgical site infection.

The sample required statistically to compare between two proportions was 6.8 or 7 rats. Another 10-20% was added to overcome possible drop outs.

The material used in this study were liophylized amniotic membrane (Dr. M Jamil Hospital tissue bank) and tissue glue (hystoacryl, B-braun, Germany). The tools utilized in this study were: Nerve stimulator (Stimuplex, B-Braun, Germany), Loop (Optivisor, USA) and Digital multimeter UX 838TR (Heles, China).

The rats in the treatment group received surgery at the right rear thigh with the left remained free, while those in the control group received surgery at the left rear thigh with the right thigh free. The sciatic nerve of the animal subjects were cut and a gap of 5 mm was made, both the distal and proximal stumps were fixated to surrounding tissue using 6-0 or 7-0 polypropilene stitches. The treatment group had a nerve conduit placed over the gap using premade human amniotic membrane fixed using cyano-acrylate glue (hystoacryl). Each of the nerve ends insert into the conduit for a minimum of 2 to 2.5 mm.

To make the nerve conduit using liophylized amniotic membrane, a sample of the nerve was taken and the diameter estimated. The diameter of the nerve conduit was made 20% larger than the estimated nerve size, about 1.8 to 2 mm. The nerve conduit was made using amniotic membrane as many as 3 layers with the needle as a template, then it was fixed in place using tissue glue (histoacryl).

At the 21st day a conduction test was performed on the samples and then a 10 mm segment of the sciatic nerve including the gap covered by nerve conduit was biopsied and examined by a Pathologist. (Figure 1)

The data gathered based on the conduction test was data containing electrical flow which was read of the ampere meter after a 3 Ampere flow was produced by the nerve stimulator. The data was then calculated the percentage compared to the initial current, if the current increased at least 25% from the initial current then it was a positive conductive tent. The data obtained through histopathology examination was nerve/axonal growth towards the distal gap, to investigate radial nerve growth and an inflammatory reaction around the conduit.

To determine nerve growth towards the distal gap, we histopathologically evaluate the presence of neural cells at the distal gap. If one or more neural cell was discovered, then the results were deemed as positive. The radial nerve growth can be estimated using histopathological examination by finding the presence of neural cells which grow out of the myelin sheath. Such findings were deemed positive result. Inflammation reaction around the nerve conduit was evaluated by determining the number of leucocyte in five fields with 400x magnification, defined as no reaction (no leukocyte per view), minimal (0-50 leukocytes per view), moderate (50-100 leucocytes per view); and extreme (above 100 leukocytes per view).

An analysis was performed using non-parametric statistical analysis Sign test using SPSS 18.

This study have received ethical clearance from the Ethical Board of Health research from Faculty

Table 1. Cnduction test results

No. Rats	Electric current from nerve stimulator (A)	Electric current recorded on ampere meter (A)	Percent (%)	+/-
1 (control)	3	0	0	-
2 (intervention)	3	0.98	32	+
3 (control)	3	0	0	-
4 (intervention)	3	1.32	44	+
5 (control)	3	0	0	-
6 (control)	3	0	0	-
7 (intervention)	3	1.65	55	+
8 (intervention)	3	1.74	58	+
9 (intervention)	3	0	0	-
10 (intervention)	3	1.25	41.6	+
11 (control)	3	0	0	-
12 (control)	3	0	0	-
13 (intervention)	3	1.11	37	+
14 (intervention)	3	0.89	29.6	+

of Medicine Universitas Padjadjaran/Hasan Sadikin hospital.

Results

After 21 days of observation, all study subjects survived without postoperative infection. The conduction test showed that all samples in the treatment group obtained positive results as shown in table 1. Non parametrical statistical analysis using the Sign test obtained a probability for the conduction test as 0.026 ($p<0.005$). There was a significant difference between the treatment group using liophylized amniotic membrane and the control group.

The nerve end test at the distal gap of the nerve conduit were analyzed using the Sign test, revealed that the probability as many as 1, therefore we concluded that there was no difference between the treatment and control group. At both groups we found minimal inflammatory reaction, and the number of leukocytes 0-50 leukocytes per view. (Table 2)

Discussions

In this study, there was no intervention for the control group because if the control group treatment using the gold standard of reconstructing gap (autograft nerve) from other nerve which has adequate or same diameter to gain an optimal result in nerve repair, such as opposite sciatic nerve, it will increase the morbidity and also the mortality of the animals. The second was because of difficulty in getting of the fabricated conduit and the price was very expensive.

The Parameter to evaluate motoric nerve recovery besides of using conduction test was the contraction of muscles that expressed by ankle range of motion. But because of the limitation of observation time, so we only observed until the nerve pass the gap.

The conduction test revealed that all there was a significant difference between the treatment and control group. The conduction test was consistent with the histopathological findings showing neural cells at the distal part of the gap, showing good result and significant difference between the treatment and control groups. This was similar to previous studies that have shown that by the 3rd week non-myelinated axons were found to have crossed a 10 mm gap.[16] Furthermore, it was seen that the nerve conduit can inhibit infiltration of fibrotic scar tissue that may block the gap between both ends of the nerve which will in turn inhibit axonal regeneration.

Table 2. Histopathological assessment results

No. Rats	Nerve Cell at Distal Gap	Nerve Cell Outside Nerve Conduit	Average Number of Lecocyte/high power field
1 (control)	No	Yes	5 (min)
2 (intervention)	No	Yes	7 (min)
3 (control)	No	Yes	10 (min)
4 (intervention)	No	No	9 (min)
5 (control)	No	Yes	6 (min)
6 (control)	No	Yes	8 (min)
7 (intervention)	Yes	No	6 (min)
8 (intervention)	Yes	No	8 (min)
9 (intervention)	No	Yes	5 (min)
10 (intervention)	Yes	No	7 (min)
11 (control)	No	Yes	4 (min)
12 (control)	No	Yes	5 (min)
13 (intervention)	Yes	No	7 (min)
14 (intervention)	Yes	No	9 (min)

Histopathological examination revealed that only one sample in the treatment group showed radial axonal growth, which was significantly different to the control group. (Figure 2) This shows that liophylized amniotic membrane was effective as a nerve conduit that will help guide axonal growth from the proximal stump to the distal stump of the cut nerve ends and inhibit fibrotic tissue infiltration that may interfere with axonal regeneration.[17]

The one sample in the treatment group that had radial axonal growth was most likely caused by a defect in the amniotic membrane handmade tubular nerve conduit due to the fragility of the material. This problem may be avoided by making the handmade tubular nerve conduit from liophylized amniotic membrane c were fully to avoid damaging the fragile material, making thick layers, and using tissue glue.

Tissue reaction to the handmade tubular nerve conduit made from liophylized amniotic membrane was nil when examined macroscopically, and this was consistent up to the 21st day when we found no signs of inflammation in the surgical wound or the tissue around the nerve conduit. This was in concordance with the histopathological examination which showed only minimal inflammatory reactions around the nerve conduit for all samples. This finding shows that liophylized amniotic membrane as a nerve conduit only causes minimal inflammatory reaction and possess low antigenicity, and this was important for biocompatibility with the surrounding tissue.

Conclusions

We concluded that handmade tubular liophylized amniotic membrane was effective to be used as a nerve conduit in the repair of peripheral nerve damage in rats with a gap of 5 mm.

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