Clinical Research

Histological evaluation of sciatic nerve anastomosis wrapped by freeze dried human amniotic membrane in rat sciatic nerve defect model

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ABSTRACT

Introduction: In peripheral nerve injury, poor environment condition, such as excessive inflammation and scar formation surrounding an injured nerve, will prevent nerve regeneration process. Human amniotic membrane with low imunogenicity has proven to decrease fibrous formation in myocardial infarction and liver fibrosis in animal models. Recent studies showed that the human amniotic membrane can suppresses the expression of the potent pro-inflammatory cytokines.

Methods: This research was conducted on thirty Sprague Dawley rats and divided into 2 groups. In control group, the sciatic nerve was totally transected and repaired by epineural microsutures. In the experimental group, after the anastomosis of injured nerve, the nerve was wrapped using freeze dried human amniotic membrane. Subsequently, it was evaluated using Hematoxylin Eosin and Mason's Trichrome staining on day 7th, 14th and 21st after anastomosis and then converted into inflammation and fibrosis score.

Results: Freeze dried human amniotic membrane decreases the inflammation score of the injured nerve only in the first week after anastomosis procedure ($p<0,05$). However, there was no significant difference of fibrosis score during 3 weeks after application.

Conclusion: Freeze dried human amniotic membrane decreases inflammation reaction within the first week after anastomosis in Sciatic nerve defect model.

ABSTRAK

Pendahuluan: *Pada cidera saraf tepi, lingkungan di sekitar cidera dapat mempengaruhi proses regenerasi seperti reaksi inflamasi yang berlebihan dan pembentukan jaringan parut disekitar saraf yang mengalami cidera.* Human Amniotic Membrane *(HAM) yang bersifat imunogenesitas yang rendah, telah terbukti dapat mengurangi pembentukan jaringan parut pada kasus infarkmiokard dan fibrosis hati pada penelitian hewan coba. Pada penelitian terbaru didapatkan bahwa HAM dapat menekan ekspersi dari sitokin pro-inflamasi.*

Metode: *Penelitian ini menggunakan 30 tikus Sprague Dawley, yang dibagi atas 2 kelompok. Pada kelompok kontrol, kami melakukan pemotongan secara total nervus Ischiadikus yang kemudian dilakukan penjahitan mikro (epineural). Pada kelompok uji coba, setelah dilakukan penjahitan kemudian dibalutkan* freeze dried human amniotic membrane*. Setelah itu dilakukan evaluasi histologist menggunakan pewarnaan* Hematoxylin Eosin *(HE) dan* Mason's Trichome *pada hari ke 7, 4 dan 21 setelah dilakukan penjahitan. Evaluasi histologist dikonversikan menggunakan sistem skoring inflamasi dan fibrosis.*

Hasil: *Pada kelompok uji coba didapatkan penurunan skor inflamasi secara bermakna pada minggu pertama setelah penjahitan ($p<0.05$). Sedangkan pada skor fibrosis tidak didapatkan perbedaan yang bermakna.*

Kesimpulan: *Penggunaan* freeze dried human amniotic membrane *sebagai pembalut saraf yang mengalami cidera, terbukti dapat menurunkan reaksi inflamasi pada minggu pertama setelah dilakukan penjahitan pada uji hewan coba.*

Keywords: peripheral nerve injury, freeze dried human amniotic membrane, inflammation score, fibrosis score

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INTRODUCTION

In peripheral nerve injury, even after early and proper primary repair of the injuried nerve, regeneration may not proceed withoptimal results.Most of the patientsare usuallyin their active age whoneed better function after treatment.[1,2]Approximately 30% cases of peripheral nerve injury is laceration injury. The restcould be compression injury, traction injury, vibration or electrical injury.[2]In case of total transection of peripheral nerve injury, the gold standard of treatment was direct nerve repair. However, the outcomes after repair are still affected by many factors, such as inflammation reaction and fibrotic tissue formation.[3,8]

In many studies, the human amniotic membrane (HAM) is reported to have anti-inflammatory, antifibrotic, anti-adhesion and antibiotics effects with low immunogenecity.[4,6,7]The human amniotic membrane preparation usedmaybe fresh, dehydrated,orfreeze dried. The freeze dried HAMretained most of the physical, biological, and morphologic characteristics of amniotic membrane (AM), reduces the preservationcosts& can be stored in a long term period.[5] This study is aimed to evaluate the inflammatory and fibrotic formation during nerve regeneration after application of freeze dried human amniotic membrane according to histological scoring system .

METHODS

Thirty healthy Sprague Dawley Rats weighing approximately 300 gr were divided into 2 groups (n=15). All rats werekept and fed according to the standardof LPPT Gadjah Mada University, Yogyakarta. The freeze dried human amniotic membrane was produced by Tissue Bank Unit of Dr. Soetomo General Hospital, Surabaya.

All animals were anesthetized by intramuscular administration of ketamine-xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Animal Ethics Committee of the Gadjah Mada University. The right sciatic nerve was exposed through a gluteal muscle incision and sciatic nerve was totally transected with a knife no 1 and anastomosis with 8/0 monofilament non-absorbable suture. Afterwards, the muscle was sutured with resorbable 4/0 sutures, and the skin was closed with 3/0 nylon. In the control group, the sciatic nerve only repaired without any specific treatment. However, in the experimental group, after being repaired, the nerve was wrapped with freeze-dried human amniotic membrane. After the expected days of evaluation reached, all animalswere evaluated,anesthetized, and euthanized with cervical dislocation technique for further histopathology evaluation.

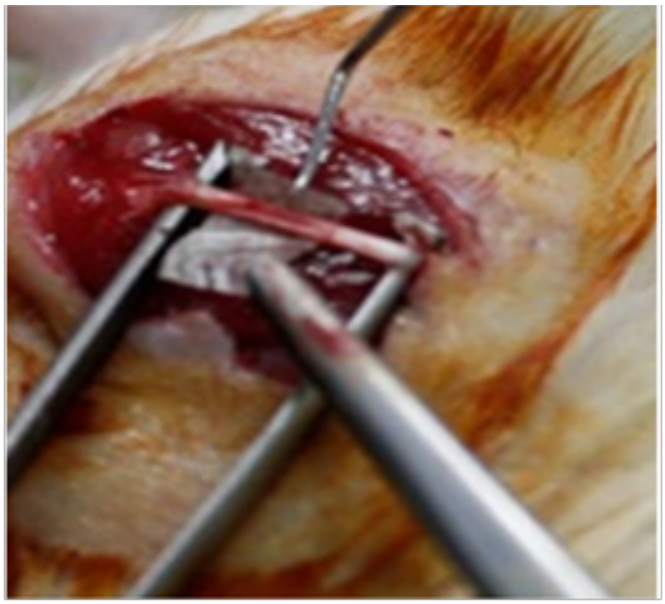

Figure 1. Wrapping freeze dried HAM after direct repair

We evaluate the histopatology on the 7th, 14th and 21stday after anastomosis. The nerve in the area of repair of the control and experimental groups were harvested. They were fixed in 10% formaldehyde, dehydrated through an ethanol series, and embedded in paraffin. The nerves were cutin 4 µm sections in longitudinal plane and then stained with Hematoxylin-eosin for general evaluation and Masson's Trichrome for fibrous collagen tissue evaluation.These were then convertedinto a scoring system by Ersoyet al.

Thepresence of inflammation was scored semiquantitatively as follows: score 0, no inflammation; score 1, mild inflammation (i.e., only a few scattered inflammatory cells were identified); score 2, moderate inflammation (i.e., small groupsof inflammatory cells in many high-power fields); score 3, severe inflammation (i.e.,many inflammatory cells either in a diffuse pattern or in large groups). The presence of fibrosis was evaluated semiquantitatively from Masson'strichrome-stained sections: score 0, small scattered areas of green staining; score 1, thin bands of green staining; score 2, thicker, connected bands of green staining; score 3, thick and dense areas of green staining.[8]

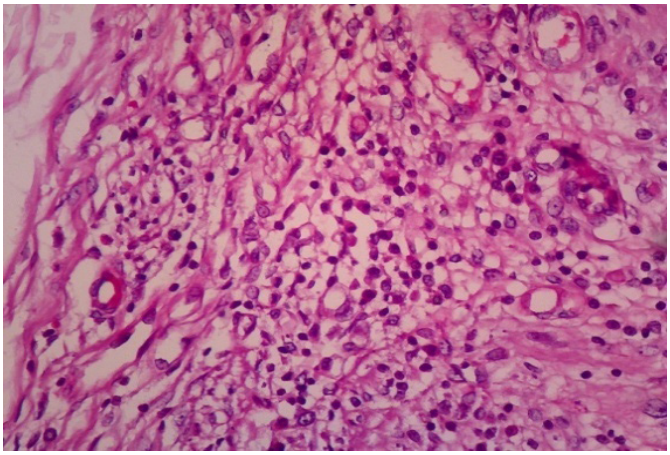

Figure 2. HE Stainingon day 21st (control group)

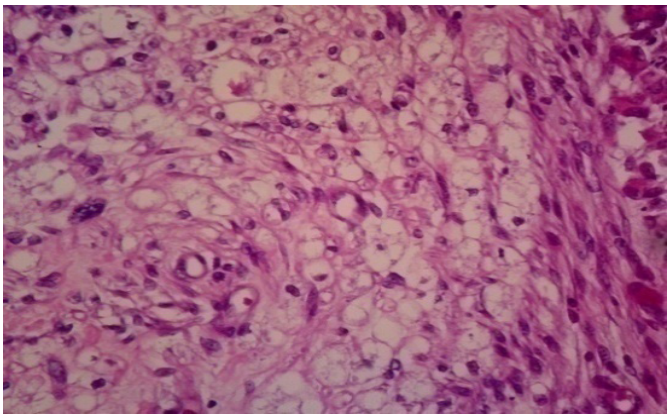

Figure 3. HE Staining on day 21st(experimental group)

RESULTS

According to the inflammation scoring system, the mean scores of the control group in all evaluation days were 3.0. In experimental group, the mean scoreswere 1.8, 1.6 & 1.6 (day 7th, 14th and 21st), resepectively. Statisticalsignificantdifference between the twogroups wasonly found inthe first week after anastomosis.

Day	Mean ±SD Control Group	Mean ± SD Experimental Group	P
7	3.0	2.8 ± 0.54	0.374
14	3.0	2.8 ± 0.54	0.374
21	3.0	2.4 ± 0.54	0.070

Table 1. Inflammation Score

According to the fibrosis score, the mean scoresof the control group in all days of evaluation were also 3.0. In experimental group, the mean scoreswere2.8, 2.8 & 2.4 (day 7th, 14th and 21st), respectively. Statisticalsignificant differencewas not found between the two groups in all days of evaluation.

Day	Mean ±SD Control Group	Mean ± SD Experimental Group	P
7	3.0	2.8 ± 0.54	0.374
14	3.0	2.8 ± 0.54	0.374
21	3.0	2.4 ± 0.54	0.070

Table 2. Fibrosis score

DISCUSSION

Nerve regeneration process is affected by several factors, such as inflammation reaction and scar formation either in between the affected areas or surrounding them.[3,8] The amniotic membrane has proven todecrease inflammatory reaction through the synthesis of anti inflammatory cytokine (IL-10 dan IL-4) and supresses the expression of transforming growth factor-β (TGFβ)andpro-inflammatory cytokine (IL-1A and IL-1B).[9]

AM alsocontains natural inhibitorof Matrix Metalloproteinases (MMPs) that expressed by the PMN and macrophage.[10]Hyaluronic acid, a heavy weight glycosaminoglycan that contained in large amount in amniotic membrane, acts as a ligand for CD44, that expressed by the inflamedcells to adhere to the stromal membrane.[11]

In our study, we found that the human amniotic membrane coulddecrease the inflammation process on injured nerve only in the first week of evaluation after anastomosis procedure. Sedighi,*et al,* in their animal study, also found decreasing inflamatory cells infiltration to the injured nerve after the 7th day, and would be significantly different on the day 21st. Zang,*et al,* also studiedthesciatic nerve in dogs and found that in the amniotic membrane group the inflammatory process was significantly lower.[12]

After nerve injury, there will be exudation of fibrin caused by pro-inflammatory cytokine. The fibroblast will be accumulated dan secreted by the extracellular matrice, especially type I an III colagen, in the affected areas. The fibroblast proliferation will induce

scarring surrounding the nerve that will cause chronic compression,sometimeseven breaking the continuity of the healing nerve. The scar can also occur intraneural that maydistrub the axonal regeneration.[3] This complex process will lead to a poor outcome of nerve healing for the patient. The amniotic membrane has proven to be able todecrease the transforming growth factor-β (TGF-β) regulation and inhibit the fibroblast receptor, so that the fibrotictissue formation will be diminished.[13]

In our study, we found that after three weeks of evaluation there was no significant difference after application with human amniotic membrane. Fesli,*et al*, found that amniotic membrane decreased the fibrotic collagen formation after 12 weeks of application in rats model.[6] Thus, the limitation inour study is short duration of evaluation. Also, it needs more combination withother methods (functional study, nerve conduction study, IHC) and more samples in order to get better results innerve regeneration after application of human amniotic membrane.

CONCLUSION

We conclude that freeze dried human amniotic membrane can decrease inflammation reaction within the first weekafter anastomosis in Sciatic nerve defect model. However, there isno significant difference infibrotic tissue formation after the application of freeze dried human amniotic membrane.

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