Original Article

Study of Transected Sciatic Nerve Repair by Amniotic Membrane with Betamethasone in Adult Albino Wistar Rats

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Abstract

Background: The aim of this study was to determine the effects of amniotic membrane impregnated with betamethasone on regeneration of transected sciatic nerve injury in adult albino Wister rats.

Methods: In this research, 42 male adult rats were divided into six equal groups. 1) Normal (intact) group: healthy rats without any injury; 2) Control group: sciatic nerve was cut and sutured; 3) Sham group: 0.2 mL culture medium was injected on the epineurium in the injury; 4) Amniotic membrane group (AM): Acellular amniotic membrane was used around the damaged sciatic nerve; 5) Betamethasone group (B): 0.2 mL Betamethasone (4 mg/mL) was injected in the site of damaged nerve and 6) Amniotic membrane group and Betamethasone (AM/B) group: Acellular amniotic membrane impregnated with 0.2 mL betamethasone was used around the damaged sciatic nerve. The rate of recovery was studied by Sciatic Functional Index (SFI), withdrawal reflex latency (WRL) test and electrotrophysiological assessments at 2, 4, 6 and 8 weeks after surgery. Histological assessment was done 8 weeks after surgery.

Results: At 8 weeks after surgery, SFI, WRL test and electrophysiological values in AM/B group were significantly improved compared to control and sham groups ($P < 0.05$). Histological results showed improvement in therapeutic groups, especially AM/B group compared to control and sham groups and other therapeutic groups ($P < 0.05$).

Conclusion: The present study showed the positive effects of Amniotic membrane and Betamethasone on nerve regeneration of transected sciatic nerve in a rat model.

Keywords: Amniotic membrane, betamethasone, repair, sciatic nerve

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Introduction

Peripheral nerve injury is a prevalent complication that can lead to complete functional loss or permanent impairment. Following nerve injury, nerve fibrosis and scar formation are thought to interfere with growth, cause deformities, and impair normal function.[1] Scar formation at the injury site of peripheral nerves creates a mechanical barrier to the sprouting axons and might inhibit axonal regeneration. Thus, scar formation has a negative impact on the degree of functional recovery. Several previous studies have investigated the effects of a range of scar-suppressing drugs such as triamcinolone acetonide,[2] triamcinolone hexacetonide,[3] collagenase,[4] hyaluronic acid-carboxymethyl cellulose membrane,[5] aprotinin,[6] human amniotic fluid,[7] low-dose external beam radiation,[8] tissue plasminogen activator,[9] or melatonin[10] at the site of peripheral nerve injury. However, few studies have achieved satisfactory restoration of function. Amniotic membrane (AM) is a layer of bio-membrane between mother and the fetus. Nerve wrapping with the AM, an effective material on nerve regeneration exhibited good biological characteristics such as convenient use, biocompatibility and low antigenicity.[11–20] It likely forms an enclosed regeneration chamber to isolate the nerve from surrounding tissue, thus reducing invasion of fibrous tissue and inflammatory cells and effectively preventing formation of anastomotic scar tissue. Furthermore, amniotic membrane matrix inhibits fibrosis and stimulates fibroblasts to produce collagen and extracellular matrix components. Furthermore, it plays a basilar membranous role to clear the obstacles against the regeneration of the nerve fibers.[21] As a result, AM can be used as a scaffold for tissue engineering in the field of neuroscience. For the first time, Mohammad, et al. (2000) used AM as a peripheral nerve conduit;[20] since then, a number of studies have been conducted on the subject. Mligiliche, et al.[19] reported that a catheter of 1 – 2 mm diameter of de-epithelized AM had the best repair effect. Mohammad, et al.[20] reported that AM had better neural regeneration effect in rat sciatic nerve defects.

Steroid medication is used for inhibition of inflammatory response and consequently recruitment of macrophages. The presence of such cells close to the site of damage and along an injured rat sciatic nerve has been shown to accelerate nerve regeneration in short term (6 days).[22,23] Also, steroids inhibit lipid peroxidation and retard both antegrade and retrograde nerve degeneration after peripheral nerve injury. Thus, they could reduce post injury dysfunction and accelerate recovery.[24,25] Nasser, et al. (1996) showed that in patients treated with 21-aminosteroid, a methylprednisolone analogue, lipid peroxidation was inhibited and thus the substance has a protective effect on the crush injury

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of the nerve.[26] Al-Bishri, et al. (2005) found that administration of Betamethasone has beneficial effects on the functional recovery of the crushed sciatic nerve injury.[27] Treatment with methylprednisolone reportedly had a significantly better improvement in sensation and motor function.[28]

The purpose of this study was to investigate the effects of a cellular amniotic membrane impregnated with Betamethasone applied for repair of transected sciatic nerve injury in albino Wister rat.

Material and Methods

Preparation of amniotic membrane

Human placenta was obtained from healthy pregnant women after caesarean section and other experimental procedures in accordance with the Ethics Committee of Neuroscience Research Center, Baqiyatallah University of Medical Sciences. The placenta was immediately washed with Earle's balanced salt solution (Sigma, St.-Louis, USA), and the chorion was removed, washed 3 times in PBS to remove blood and cellular debris, incubated with EDTA for 2 hours at 37°C and de-epithelized with a cell scarper. The AM was cut into 1 cm × 1 cm segments.[11]

Animals and Surgical procedure

The animals were placed in a temperature and humidity-controlled room with 12h light/12h dark and permitted free access to standard rodent laboratory food and water. In this study, 42 male adult rats were divided randomly into six groups including: 1) Normal (intact) group: healthy rats without any injuries; 2) Control group: rats with transected and sutured sciatic nerve; 3) Sham group: 0.1 mL DMEM containing 10% FBS was injected on the damaged sciatic nerve; 4) Amniotic membrane group (AM): Acellular amniotic membrane was used around the damaged sciatic nerve; 5) Betamethasone group (B): 0.2 mL Betamethasone (4 mg/mL) was injected in the site of damaged nerve; and 6) Amniotic membrane and Betamethasone (AM/B) group: Acellular amniotic membrane impregnated with 0.2 mL betamethasone was used around the damaged sciatic nerve. For sciatic nerve dissection, the right hind limb was shaved and swabbed with antiseptic solution (Betadine). All operations were performed on this limb. One longitudinal cutaneous incision was made on the posterolateral side of the thigh in length of 3 cm to expose the sciatic nerve. Sciatic nerve was transected. Interventional treatment was done. The epineurium was sutured with 7/0 Prolene sutures. The muscle fascia and skin were then sutured with 4/0 nylon sutures and swabbed with antiseptic solution.

Electrophysiological studies

At 8 weeks after surgery, rats were anesthetized and the sciatic nerves were exposed. Electric stimulation (duration of 0.1 ms, intensity of 2.3 mA) was applied to the proximal site of the injured nerve. The compound muscle action potential as these can be considered to reflect the amount of activated fibers was recorded in the gastrocnemius with a needle electrode and a reference cap electrode inserted at the knee joint. The stainless steel needle used as the ground electrode was inserted into the tail skin.

Functional assessment of nerve regeneration

Walking track analysis was carried out 2, 4 and 6 weeks after surgery based on the method of Bain, et al.[23] The rats were permitted conditioning trials in a 60 × 7 × 20 cm walking track with a piece of white paper at the bottom of the track. The hind feet were dipped in ink, leaving prints on the white paper. The lengths of the third toe to its heel (PL), the second toe to the fourth toe (IT), and the first to the fifth toe (TS) were measured on the contralateral normal side (N), and the experimental side (E) in each rat. The sciatic functional index (SFI) of each animal was computed by the following formula:

SFI= |-38.5 (EPL-NPL/NPL) + 109.5 (ETS-NTS/NTS) + 13.3 (EIT-NIT/NIT)-8.8|

In this study, SFI oscillates around 0 for normal nerve function, whereas around 100 SFI represents total motor sciatic nerve dysfunction.

Withdrawal reflex latency test

For measurement of withdrawal reflex latency (WRL) at 2, 4 and 6 weeks after surgery, the rats in all groups were positioned to stand with the affected hind paw on a hotplate at 56°C. WRL is defined as the time elapsed from the onset of hotplate contact to withdrawal of the hind paw and measured with a stopwatch. The affected limbs were tested three times, with an interval of 2 min between consecutive tests to prevent sensitization, and the three latencies were averaged to obtain the final result.

Histomorphometric evaluation

At 8 weeks after surgery, the animals were killed and sciatic nerves were removed and fixed in 10% formalin for more than 24 hours. The fixed specimens were washed in normal saline, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin and cut into 5 µm thick sections and stained by Hematoxylin and Eosin (H&E) for histologic study. For each section on slide, photographs were taken with digital camera from three random fields and analyzed with motic software.

Statistical analysis

The results were expressed as means ± SEM. Statistical analyses were performed using (SPSS 22.0 software package, SPSS Inc., Chicago, IL). Statistical differences between groups were analyzed by Two-Way Repeated Measurements analysis of variance (ANOVA) [for SFI and withdrawal reflex latency] with training Weeks (Week) as within-subjects factor and experimental group (Group) as between-subjects factor or one-way analysis of variance (ANOVA) [for electromyographical and histomorphological assessments] followed by Post-Hoc Tukey for comparisons between groups. The significant differences were set at $P < 0.05$.

Result

Recovery of sciatic nerve function

Graph 1 show SFI values in all experimental groups. Prior to surgery, SFI values in all groups were near zero. After nerve transection, the mean SFI increased to 100 due to the complete loss of sciatic nerve function in all animals. At 2, 4, 6 and 8 weeks after surgery, SFI values increased significantly in experimental (control, sham, AM, B and AM/B) groups compared to intact group. At 2 weeks after surgery, improvement in SFI was found in all groups but there was no significant difference between groups. At 4 weeks after surgery, SFI values decreased significantly in

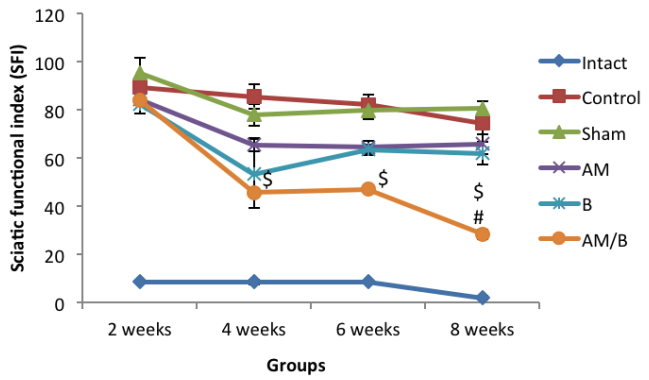

Figure 1. Comparison of the mean functional recovery of each group in term of SFI derived from walking-track prints for the six different groups measured at 2, 4, 6 and 8 weeks after surgery. (Data are shown as the mean ± SEM. $: showed statistically different with Control and Sham groups ($P < 0.01$); #: showed statistically different with AM and B groups ($P < 0.05$)).

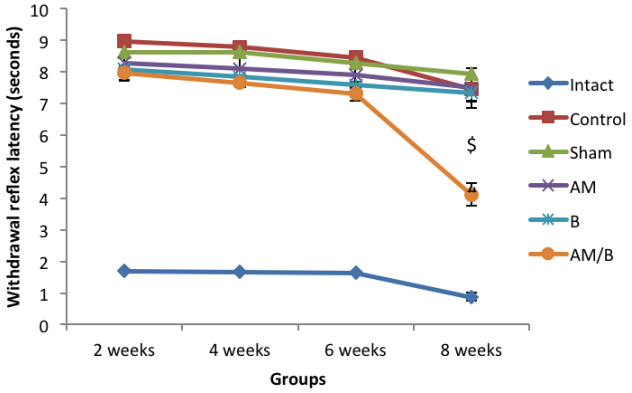

Figure 2. WRL analysis is shown at 2, 4, 6 and 8 weeks after surgery. (Data are shown as the mean ± SEM. $: showed statistically different with Control and Sham groups; #: showed statistically different with AM and B groups).

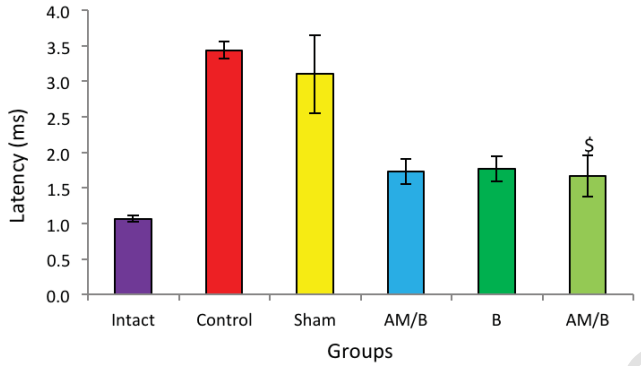

Figure 3. Comparison of mean latency (ms) analysis in all experimental groups. (Data are shown as the mean ± SEM; $: showed statistically different with control and sham group ($P = 0.005$ and $P = 0.02$)).

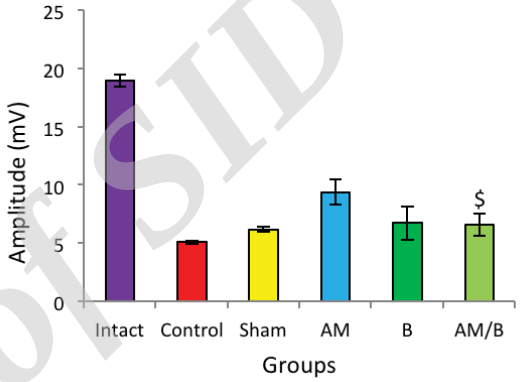

Figure 4. Comparison of mean amplitude (mV) analysis in all experimental groups. (Data are shown as the mean ± SEM; $: showed statistically different with control and sham groups ($P = 0.01$ and $P = 0.04$)).

AM/B group compared to the control and sham groups ($P < 0.05$). At 6 weeks after surgery, SFI values decreased significantly in AM/B group compared to the control and sham groups. At 8 weeks after surgery, improvement in SFI in all animals was observed again, especially in AM/B group compared to the control and sham groups [Two-Way repeated measurement ANOVA; Week effect: ($F1, 3$) = 31.971, $P < 0.001$; Cs/PEO effect ($F1, 5$) = 30.455, $P < 0.001$; hWJ-MSCs effect: ($F1, 5$) = 30.455, $P < 0.001$] (Figure 1).

Withdrawal reflex latency findings

As shown in Figure 2, at 2, 4, 6 and 8 weeks after surgery, WRL increased significantly in intact group compared to experimental groups. WRL decreased significantly in AM/B group compared to control, sham, AM and B groups at 8 weeks after surgery [Two-Way repeated measurement ANOVA; Week effect: ($F1, 3$) = 18.040, $P < 0.0001$; Cs/PEO effect ($F1, 5$) = 28.529, $P < 0.0001$; hWJ-MSCs effect: ($F1, 5$) = 28.529, $P < 0.0001$] (Figure 2).

Electrophysiological studies

Our data showed that 8 weeks after surgery, latency and amplitude were significantly different in intact group compared to the control and sham groups ($P = 0.001$ and $P = 0.01$). The recovery index of latency (ms) in AM/B group decreased significantly compared to the control and sham groups ($P = 0.005$ and $P = 0.023$). The recovery index of amplitude (mv) in AM/B group significantly increased compared to the control and sham groups ($P = 0.011$ and 0.046). Figures 3 and 4 show latency and amplitude analyses in all experimental groups.

Histological and morphometric findings

Figures 5 and 6 shows the quantitative morphometric analyses of distal segment of damaged sciatic nerves for each of the experimental groups. At 8 weeks after surgery, in the category which have more than 6 µm diameter, the number of nerve fibers decreased, although there was no significant difference between groups ($P > 0.05$). In category which have 4 to 6 µm diameter, the number of nerve fiber decreased significantly in experimental groups compared to intact group ($P < 0.001$). In this category the number of nerve fibers decreased significantly in AM compared to control ($P = 0.001$) and B groups compared to the control and sham groups ($P = 0.043$ and $P = 0.05$). In addition, there was significant decrease in the number of nerve fibers in AM/B group compared to the control and sham groups ($P = 0.004$ and $P = 0.006$). In the category which have less than 4 µm, there was significant difference between intact and experimental groups ($P < 0.001$) except for AM/B group. In this category, the number of nerve fibers decreased significantly in AM/B group compared to the control and sham groups ($P = 0.019$ and $P = 0.011$) (Figures 5 and 6).

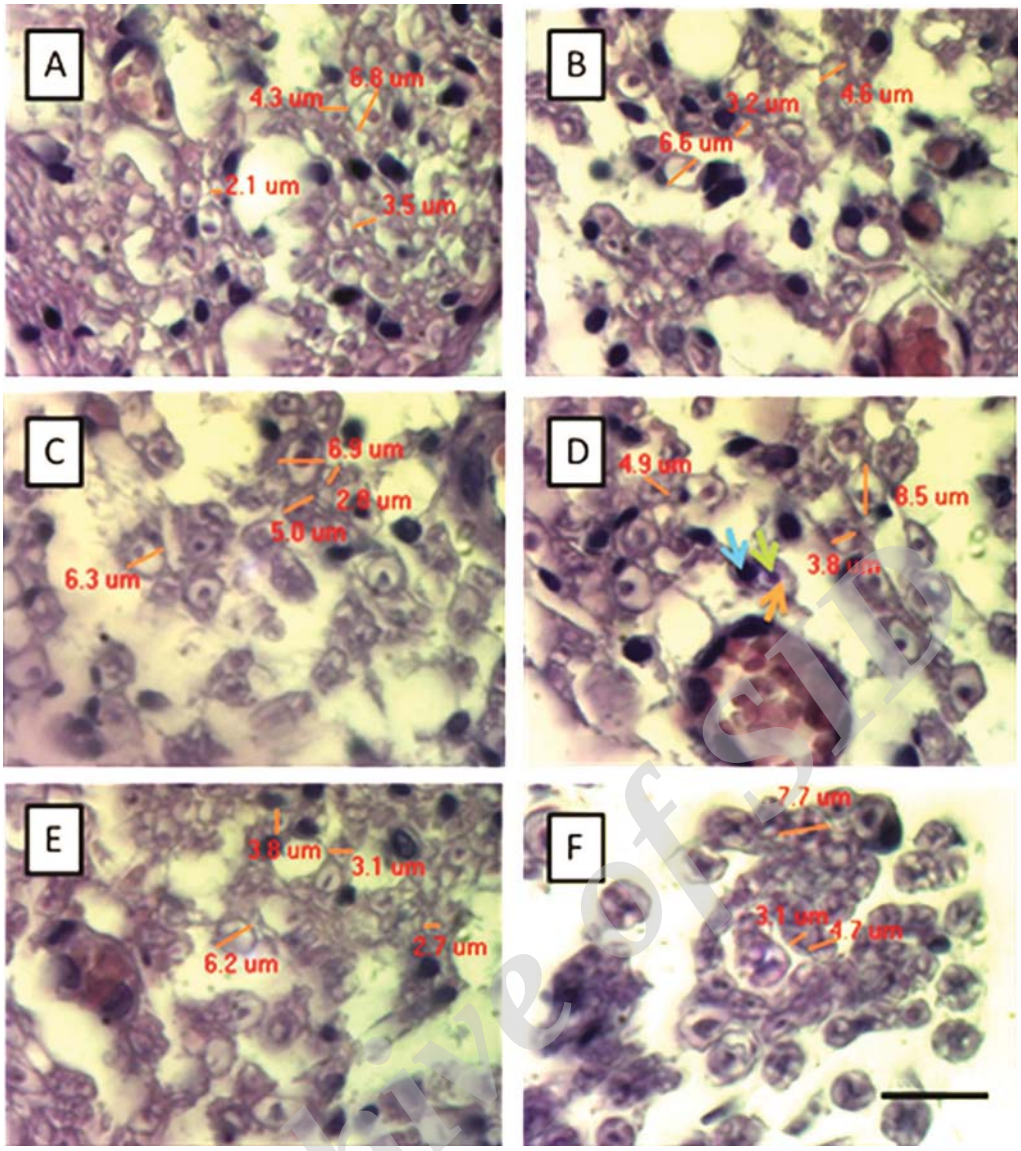

Figure 5. Cross sections through distal segment damaged sciatic nerve at 8 weeks after surgery in different groups. **A)** Intact group; **B)** Control group; **C)** Sham group; **D)** AM group; **E)** B group and **F)** AM/B group. Blue arrow indicates schwann cell, Green arrow indicates axon and Orange arrow indicates myelin sheath. Scale bar: 20 µm

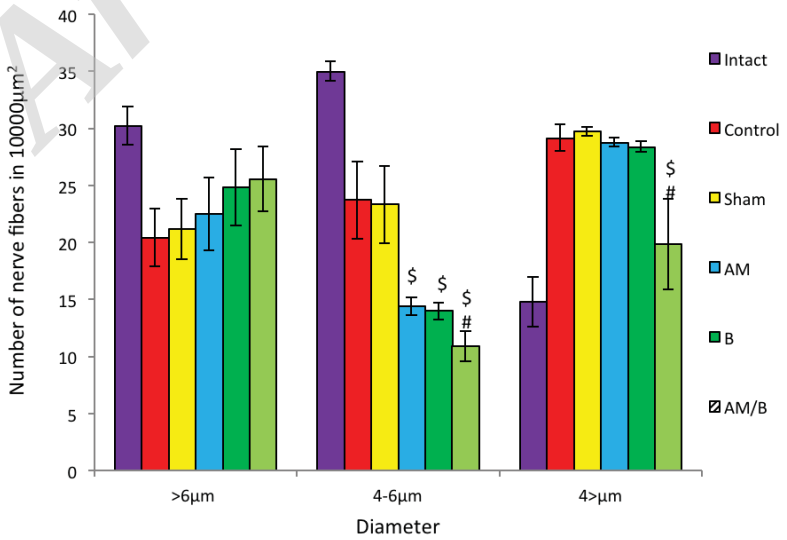

Figure 6. The number of nerve fibers is shown in different categories based on diameter. (Data are shown as the mean± SEM**;** $: showed statistically different with Control and Sham groups; #: showed statistically different with AM and B groups).

Discussion

The peripheral nervous system has the potential to regenerate after damage. However, following peripheral nerve lesion, functional recovery and successful regeneration is not always efficient, especially in neurotmesis. When peripheral nerve is damaged, nerve fibrosis is thought to interfere with growth, create a mechanical barrier to the sprouting axons and impair normal function. In other words, peripheral nervous system damage limits functional results and decreases patients' quality of life. This may impose enormous psychosocial and economic burdens on the patients and society.[29]

Human amniotic membrane has biological properties, which include easy availability, comparatively low costs of preparation, storage, and use, anti-adhesive, antibacterial, low immunogenicity, anti-inflammatory, anti-scarring properties and it mediates tissue repair via the its growth factors which enhance the healing process.[30]

On the other hand, steroid medication inhibits lipid peroxidation and decreases functional dysfunction after peripheral nerve injury. Also, lipid peroxidase inhibitors retard nerve degeneration after axotomy.

Walking track analysis is the gold standard for evaluation of nerve recovery after sciatic nerve injury because proper walking requires a coordinated activity involving sensory input, motor response, and cortical integration.[27] In the present study, there were better results in SFI of AM/B group at 8 weeks after surgery. This result was in agreement with Mohammad, at al. (2000), Forootan (2011) and Meng, et al. (2011).[15,20,31] Mohammad, et al. reported that recovery at 2 to 4 weeks was higher in amniotic group. Forootan, et al. reported that SFI improved 12 weeks after surgery. Meng, et al. showed SFI improvement was significant at 6 and 10 weeks after operation.

WRL values were used to evaluate motor performance and nociceptive function of peripheral nerves. Our data showed that A/B group was better than other groups. The present study agrees with the reports of Simos, et al. (2010), Gartner, et al. (2012) and Basiri, et al. (2013).[32–34] Gartner, et al. reported that sensory function recovered 12 weeks after surgery. Simos, et al. reported that chitosanIII induced better regeneration and functional recovery in comparison with PLGA group. Basiri, et al. reported that withdrawal response recovered after 6 weeks.

Our data showed that at 8 weeks after surgery, the recovery index of latency and amplitude improved in therapeutic groups, especially in AM/B group compared to the control and sham groups. These results agree with Henry. (2009), Forootan, et al. (2011) and Zhang, et al. (2013).[31,35,36] Henry, et al. (2009) demonstrated that amniotic membrane wrapping had better electrophysiological results in nerve regeneration. Forootan (2011) reported that amniotic membrane had better results in nerve regeneration. Zhang (2013) reported that amplitude significantly improved in amnion-wrapped allogenic nerve at 16 weeks.

In histologic studies, quantitative morphometric indices of regenerated nerve fibers showed improvement in AM/B group indicating a beneficial effect of amniotic membrane impregnated with betamethasone on nerve regeneration. These results agree with Meng, et al. (2011), Zhang, et al. (2013) and Mohammadi, et al. (2013).[15,37,38] Meng, et al. (2011) showed that AM wraps significantly increased the number of myelinated axons at of 8 weeks after surgery. Zhang (2013) reported that amnion-wrapped allogenic nerve transplantation showed better regeneration at 16 weeks and might improve defected nerve morphology and the quality of transplanted nerve regeneration. The neurite density was significantly improved in amnion-wrapped allogenic nerve transplantation group. Mohammadi, et al. (2013) showed that the number of myelinated fibers were significantly higher in the betamethasone group.

The mechanism by which AM might improve recovery is complex. Amniotic membrane is an extraembryonic tissue composed of three main layers: an epithelium layer, a thick basement membrane and a vascular mesenchyme. The exact mechanism of anti-inflammatory effects of amniotic membrane is not known. It is assumed that AM act as a barrier to prevent the infiltration of inflammatory cells into the affected area, thus reducing inflammatory mediators. Also, it is reported that it entraps T lymphocytes.[39]

Fibroblasts are responsible for the formation of scar fibroblasts during wound healing and are activated by transforming growth factor beta. Amniotic membrane inhibits TGF-β receptor expression in fibroblasts and causes less fibrosis.[40] On the other hand, amniotic membrane is a suitable substratum for the growing axons in the central nervous system.[41]

Following nerve injury, inflammatory reaction to trauma is triggered by increased vascular permeability and edema. Steroids as anti-inflammatory agents prevent inflammation by inhibiting phospholipase A2. This action is possible by inhibition of degranulation of granulocytes, mast cells or macrophages and by suppressing macrophage inhibitory factor and lysosomic and other membrane stability. Steroids are used in the treatment of peripheral nerve injury. In fact, phospholipase is an enzyme responsible for production of inflammatory agents and may be involved in the process of myelin degeneration in peripheral nerves seen in Wallerian degeneration of peripheral nerves.[38]

In conclusion, this study showed that AM wrapping impregnated with betamethasone of the sciatic nerve enhanced functional recovery and nerve regeneration which may be useful for the clinical outcome improvement that is often unsatisfactory and there is rarely a complete return of function.

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