Assessment of processed human amniotic membrane as a protective barrier in rat model of sciatic nerve injury

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A B S T R A C T

Following nerve injury, scar formation is thought to be a considerable impediment to axonal regeneration at the nerve injury site. Nerve wrapping can protect the regenerating axons, and human amniotic membrane (HAM) derived from human placenta is an effective material for that purpose. The impact of nerve wrapping with HAM on functional recovery after nerve injuries, especially after autograft repair of long gap lesions, has not been comprehensively investigated. In the current study, we investigated whether the application of HAM as a nerve wrap to a 10 mm segment of transected and repaired nerve would reduce scar formation and permit better axonal regeneration and/or functional recovery in rats. The outcome was assessed with morphological and functional measures. We found that nerves wrapped with HAM had significantly fewer adhesions and less scar formation than controls. Although the final outcome, both functionally and morphologically, was not significantly improved by wrapping the nerve with HAM, the observed decrease in adhesions and scar formation might help the nerve retain its mobility and thus prevent traction injury and ischemia, which are caused by nerve tethering to the adjacent tissue during the healing process.

Peripheral nerve injury with long gap lesions results in partial or complete loss of nerve function. Clinically, implantation of an autologous nerve graft is a standard technique to repair peripheral nerve gaps; however, this “gold standard” procedure has several limitations. Recently, a range of synthetic and natural materials have been studied as alternatives for peripheral nerve repair, but few studies have achieved satisfactory restoration of function. The lack of functional recovery is probably multifactorial. The amount of scar formation at the injury site of peripheral nerves has a negative impact on the degree of functional recovery [3]. The formation of perineural and intraneural scarring might inhibit axonal regeneration because scarring interferes with growth, causes deformities, and impairs normal function [11]. Therefore, it is imperative to reduce scarring to promote functional recovery after nerve injury.

Previous studies have investigated the effects of a range of antiscarring agents and of different approaches at the injury site. Applying agents, such as triamcinolone acetonide [7], triamcinolone hexacetonide [17], aprotinin [6], human amniotic fluid [13], or melatonin [16], to the damaged nerve reduces scarring after nerve injury. However, most of these agents are still experimental. Studies on the effect of HAM on nerve regeneration have been performed only in animal models of transected injury [14], but the inherent regenerative capacity of the nerves in animals could be sufficiently efficient over short nerve gaps that the effects of HAM might not be adequately assessed. Thus, a longer nerve gap is required to better evaluate the impact of HAM on functional recovery after nerve injury.

The current study hypothesized that nerve wrapping with HAM will reduce scar formation and benefit the functional recovery of peripheral nerves after repair of long gap lesions. We have therefore evaluated the effect of a HAM wrap on a long sciatic nerve defect (10 mm) that was repaired with an autologous nerve graft. The sciatic function index (SFI) was assessed over a 12-week period after nerve injury and surgical repair. An electrophysiological test was used at the end of the experimental period to measure functional recovery, and morphological analysis was performed to investigate the effect of HAM wraps on nerve regeneration. We found that HAM-wrapped nerves developed fewer adhesions and showed significantly less scar formation than unwrapped nerves. Although the final outcome, both functionally and morphologically, was not significantly improved by the HAM wraps, the observed decrease in adhesion and scar formation might help maintain the mobility of the nerve and thus prevent traction injury.
and ischemia, which are caused by nerve tethering to the adjacent tissue in the recovery process.

Human placenta was obtained from healthy pregnant women after caesarean section in accordance with the Declaration of Helsinki and with the approval of the institutional ethics committee. The placenta was immediately washed with Earle's balanced salt solution (Sigma, St.-Louis, USA), and the chorion was removed. The HAM was cut into 20 mm segments and stored at –80 °C in medium consisting of a 1:1 solution of 100% glycerol and Dulbecco's modified Eagle's medium (Gibco, Canada) with 1% penicillin–streptomycin. Immediately before use, the membranes were washed in phosphate-buffered saline (PBS) for 30 min and then rinsed three times [12].

All animals were obtained from the Animal Centre of the Fourth Military Medical University, and procedures were approved by the administration of the Committee of Experimental Animals in Shaanxi, China. Thirty-six male Sprague Dawley (SD) rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The left sciatic nerve was exposed, and a 10-mm segment of sciatic nerve was reversed 180° and reconnected to the proximal and distal nerve stumps with 10-0 epineural sutures. The rats were randomly divided into two groups (HAM-wrapped and unwrapped,control), and in the HAM-wrapped group, a 14 mm × 6 mm segment of HAM was wrapped around the autograft segment. The HAM overlapped each repair site by 2 mm and was secured to the epineurium of both the proximal and distal nerve stumps with 10-0 epineural sutures. The rats were then re-anesthetized, and the surgical area was gradually cleaned by microdissection. Sciatic nerves were re-exposed and evaluated to investigate the extent of fibrous tissue surrounding the repair site. Both nerve adherence to the surrounding muscle tissue and separability were assessed by a numerical grading scheme [15]: (Grade 1) no dissection or mild blunt dissection, (Grade 2) some vigorous blunt dissection required, and (Grade 3) sharp dissection required.

Electrophysiological tests were performed every 4 weeks post-operation. After macroscopic evaluation, a bipolar stimulating electrode was placed under the sciatic nerve at a location 5 mm proximal to the autograft. Recordings were conducted using superficial electrodes and performed with an electromyogram (Keypoint 4C Dantec, Denmark). The peak amplitude of the compound muscle action potential (CMAP), CMAP latency of onset, and nerve conduction velocity (NCV) values were calculated.

After harvesting nerve explants, transverse and longitudinal sections (5 μm) were cut and stained with picrosirius red (specific for collagen types I and III). The sections were analyzed using polarised light microscopy. Photographs were taken and analyzed with an image analysis system (Leica QWin software package) to measure the thickness of the scar and nerve tissue, exclude the epineurium and HAM wrap, and calculate the percentage of area of staining. The scar formation index was obtained by dividing the value of the thickness of the scar tissue by the value of the thickness of the nerve tissue. An average value from three sections was taken to represent the collagen level in each nerve.

At 4, 8, and 12 weeks post-surgery, the nerve explants were harvested and fixed in 3% glutaraldehyde. The samples were post-fixed in 1% osmium tetroxide solution for 1 h and then dehydrated and embedded in epoxy resin. Transverse semi-thin (thickness: 1 μm) and ultra-thin (thickness: 50 nm) sections were cut from the distal portion of the samples. The semi-thin sections were stained with a 1% toluidine blue/1% borax solution and were examined using a light microscope (AH3, Olympus, Tokyo, Japan). Ultra-thin sections were stained with uranyl acetate and lead citrate and were examined under a transmission electron microscope (TEM, H-600, Hitachi, Tokyo, Japan). Morphometric analyses, (a) the thickness of myelin sheath, (b) the total axon number, (c) the diameters of the myelinated nerve fibers, and (d) the G ratio, were then accomplished using the Leica QWin software package.

Student’s t tests and one-way analyses of variance (ANOVAs) for repeated measures followed by Tukey’s post hoc tests were used to compare mean values using the SPSS16.0 software package (SPSS Inc., Chicago, IL, USA). Statistical significance was accepted at the p < 0.05 level.
Table 1
Results of nerve adherence and scar formation.

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<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
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<tbody>
<tr>
<td></td>
<td>Autograft</td>
<td>Autograft + HAM</td>
<td>Autograft</td>
</tr>
<tr>
<td>Scar formation index</td>
<td>0.26 ± 0.05</td>
<td>0.19 ± 0.03*</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>Nerve adherence and separability</td>
<td>2.9 ± 0.2</td>
<td>1.5 ± 0.1*</td>
<td>2.7 ± 0.3</td>
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</table>

* p < 0.05 for comparison with autograft group.

Table 2
The electrophysiological values in two groups at predefined time post surgery.

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<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Autograft</td>
<td>Autograft + HAM</td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>1.10 ± 0.15</td>
<td>1.83 ± 0.22</td>
<td>1.77 ± 0.13</td>
</tr>
<tr>
<td>Peak amplitude (mV)</td>
<td>16.70 ± 1.73</td>
<td>5.05 ± 0.31</td>
<td>5.62 ± 0.58*</td>
</tr>
<tr>
<td>Conduction velocity (m/s)</td>
<td>34.53 ± 2.08</td>
<td>16.86 ± 1.62</td>
<td>17.29 ± 1.58</td>
</tr>
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* p < 0.05 for comparison with autograft group.

The function of the gastrocnemius muscle was evaluated by walking track analysis. As revealed by the postoperative changes in the SFI, functional performance in the two groups was impaired to the same extent one week after surgery. After the second week, the rate of reversal of the injury-induced disability was considerably faster in the HAM-wrapped group than that in the control group. However, the improvement in the SFI was only significant in the sixth and tenth weeks after injury (p < 0.05). The maximal degree of recovery in the HAM group was achieved approximately two weeks earlier than in the control group (Fig. 2).

After walking track analysis, the sciatic nerve was exposed through the original incision. There was no sign of infection or inflammatory reaction at the surgical site. The control group exhibited dense scar formation surrounding the repaired nerve. In contrast, nerves wrapped with HAM had significantly less nerve adherence to surrounding tissue and more separability compared with the control group (Table 1).

Electrophysiological studies were performed to further investigate the beneficial effect of HAM wraps on functional recovery. The peak amplitude of the CMAP, CMAP latency of onset, and NCV were calculated for each rat (Table 2). Nerve wrapping with HAM significantly lowered the CMAP peak amplitude 4 weeks after surgery (p < 0.05). Further, HAM wrapping significantly enhanced the CMAP peak amplitude and NCV 8 weeks after surgery (p < 0.05). However, the 3 parameters were not significantly different between the 2 groups 12 weeks after surgery (p > 0.05).

In the autograft controls, the mean percentage of area of staining for collagen was 7.88%. In the HAM group, the mean percentage was significantly lower, suggesting that scar formation was lower in this group (5.28%, p < 0.05) (Fig. 3).

![Fig. 3. Transverse and longitudinal sections of sciatic nerve from the control (A–D) and HAM-wrapped (E–H) groups, stained for collagen with picrosirius red. (I) Mean values of the percentage of area stained with picrosirius red in the two groups.](image-url)
Fig. 4. TEM of sciatic nerve 5 mm distal to autograft at 4 weeks (A and D), 8 weeks (B and E), and 12 weeks (C and F) after surgery. (A–C) TEM of the control group. (D–F) TEM of HAM-wrapped group. (G) Thickness of the myelin sheath measured from the distal section of the regenerated nerve for the 2 groups. *Significant differences between control and HAM-wrapped groups at the predefined time points (p < 0.05).

Analysis of ultra-thin sections of the sciatic nerve distal to the autograft revealed that regenerated axons in the HAM-wrapped nerves had significantly larger diameters than those in the control nerves 12 weeks post surgery (p < 0.05), but no significant difference in axon diameter was observed 4 and 8 weeks after surgery (p > 0.05) (Fig. 4). Further investigations showed that HAM wraps significantly increased the number of myelinated axons 8 weeks after surgery (p < 0.05). However, 12 weeks after surgery, the number of myelinated axons was similar in both the HAM wrapping and the control groups (p > 0.05). Although some beneficial effects of HAM wraps were detected, as described above, the degree of myelination estimated by the G ratio (axon to fiber diameter) was similar in both groups at the predefined time points (4, 8, and 12 weeks after surgery, p > 0.05) (Fig. 5).

Although enhanced functional recovery was observed during the early stage after surgery in the HAM group, the final outcome was not significantly enhanced by HAM wrapping of the sciatic nerve. The mechanism by which HAM might improve recovery is complex. The HAM, which is the inner layer of the fetal membranes, is composed of a thick basement membrane and an avascular stroma. It is nonimmunogenic and human amniotic cells do not express HLA-A, -B, -C, or -DR antigens of β2-microglobulin [1]. The presence of anti-inflammatory proteins has also been confirmed in HAM [8]. Clinically, the HAM has been used in a wide range of tissue repair models including urethral reconstruction,
Fig. 5. Transverse section of sciatic nerve 5 mm distal to autograft stained with toluidine blue showing regeneration of myelinated axons at 4 weeks (A and D), 8 weeks (B and E), and 12 weeks (C and F) after surgery. (A–C) Toluidine blue staining of the control group. (D–F) Toluidine blue staining of the HAM-wrapped group. The total axon number (G), diameter of the myelinated nerve fibers (H) and the G ratio (I), measured from the distal section of the regenerated nerve, were compared between the 2 groups. *Significant differences between control and HAM-wrapped groups at the predefined time points ($p < 0.05$).

and repair of the corneal [4,10]. The HAM has also been shown to be a suitable substratum for growing axons in the central nervous system [5]. In this study, wrapping an autograft with HAM isolated the coaptation point with the surrounding tissues and provided better protection to the regenerating axons. These features have led to the use of amnion, which can effectively prevent scar formation, especially perineural scarring. However, scars commonly form diffusely within transected nerves, suggesting that perineural scars are not the only ones that form; it has been noted that the amount of intraneural scarring varies with the severity of nerve trauma. It is possible that both the perineural and intraneural scars could impede nerve regeneration and negatively impact functional recovery after surgery. Thus, reducing scar formation at the repair site is associated with better functional recovery. However, the HAM wraps mainly prevent perineural scar formation and adhesions. Intraneural scar formation still impedes nerve regeneration and functional recovery. Therefore, reducing both perineural and intraneural scars might ultimately enable more
complete functional recovery. The development of new therapeutic agents that reduce intraneural scarring, used in combination with HAM wraps to reduce perineural scarring, might effectively promote nerve regeneration and allow complete functional recovery.

In conclusion, this study showed that HAM wrapping of the sciatic nerve enhanced functional recovery and nerve regeneration during the early stage after surgical injury and repair. Although HAM wraps did not significantly improve the final outcome, the observed decrease in adhesions and scar formation might allow the nerve to retain its mobility and might prevent the traction injury and ischemia caused by tethering of the nerve to the adjacent tissue in the healing process. These effects could have therapeutic importance in clinical settings. Reducing both perineural and intraneural scars might improve the outcome of nerve repair in larger animals, including humans.

References