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Comparison of human amniotic membrane and collagen nerve wraps around sciatic nerve reverse autografts in a rat model☆,☆☆,★

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ABSTRACT

Human amniotic membrane (hAM) and collagen nerve wraps are biomaterials that have been investigated as therapies for improving outcomes of peripheral nerve regeneration; however, their efficacy has not been compared. The purpose of this study is to compare the efficacy of collagen and human amniotic membrane nerve wraps in a rodent sciatic nerve reverse autograft model. Lewis rats ($n = 29$) underwent sciatic nerve injury and repair in which a 10-mm gap was bridged with reverse autograft combined with either no nerve wrap (control), collagen nerve wrap or hAM nerve wrap. Behavioral analyses were performed at baseline and 4, 8 and 12 weeks. Electrophysiological studies were conducted at 8, 10 and 12 weeks. Additional outcomes assessed included gastrocnemius muscle weights, nerve adhesions, axonal regeneration and scarring at 12 weeks. Application of both collagen and hAM nerve wraps resulted in improvement of functional and histologic outcomes when compared with controls, with a greater magnitude of improvement for the experimental group treated with hAM nerve wraps. hAM-treated animals had significantly higher numbers of axons compared to control animals ($p < 0.05$) and significantly less perineural fibrosis than both control and collagen treated nerves ($p < 0.05$). The ratio of experimental to control gastrocnemius weights was significantly greater in hAM compared to control samples ($p < 0.05$). We conclude that hAM nerve wraps are a promising biomaterial that is effective for improving outcomes of peripheral nerve regeneration, resulting in superior nerve regeneration and functional recovery compared to collagen nerve wraps and controls.

1. Introduction

Recovery after peripheral nerve injury can result in adverse sequelae including extraneural scar formation, epineural thickening, axonal misrouting and progressive irreversible muscle fibrosis [1]. Without timely nerve regeneration and reinnervation of distal muscle, motor endplates may sustain permanent injury with resultant loss or impaired function [1,2]. Various adjunctive treatments have been developed for use in conjunction with neurorrhaphy including nerve wraps and conduits [3,4,5,6]. Nerve wraps serve to protect healing nerves and facilitate axonal regeneration, potentially reducing epineural scarring and adhesion formation which can contribute to a suboptimal recovery outcome [7,8].

Results of preclinical studies assessing the use of nerve wraps and conduits have been inconsistent due to a lack of standardization in outcome evaluation [9]. However, preclinical and clinical studies evaluating nerve wraps and conduits composed of FDA-approved, commercially available materials including purified collagen and purified amniotic membrane (hAM) have reported successful outcomes [10,11,12]. These studies have established the safety and efficacy of each of these naturally biocompatible, FDA-approved biomaterials for applications in peripheral nerve repair [10,11,12]. Collagen nerve conduits in which a collagen tube bridges a gap between a severed nerve have been more widely studied than collagen nerve wraps in which the collagen tube is placed around a primarily repaired nerve. Literature on the use of hAM in peripheral nerve regeneration is limited compared to investigations of collagen-based biomaterials. However, other surgical appli-

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cations of hAM have been more thoroughly studied and studies have shown clinical benefits due to its anti-fibrotic and pro-regenerative properties [13,14,15].

Collagen-derived nerve wraps are composed of semi-permeable purified type II collagen matrices with a demonstrated ability to reduce adhesions and neuroma formation [9,11,12]. hAM is derived from the inner layer of fetal membranes and is a source of stem cells including mesenchymal and epithelial stem cells. These stem cells have the potential to reduce adhesions in the setting of peripheral nerve regeneration and modulate repair mechanisms through release of neurotrophic factors [10,13]. In addition, hAM has been shown to enhance Schwann cell proliferation, upregulate expression of glial cell-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF), increase neurite outgrowth from dorsal root ganglion neurons, and hAM-derived stem cells have been shown to differentiate into neural phenotypes [16,17]. Anti-inflammatory properties of hAM allow it to overcome limitations of other types of stem cells, such as poor differentiation, inflammation-mediated graft rejection, or tumorigenicity [18].

The purpose of this experimental study was to compare collagen and hAM nerve wraps around sciatic nerve reverse autografts in a rat model and evaluate their efficacy for reducing perineural adhesions and improving outcomes of peripheral nerve regeneration. Comparative analysis of collagen and hAM nerve wraps may help guide clinical decision making for differing peripheral nerve injuries [19].

2. Materials and methods

2.1. Experimental design

The rat sciatic nerve model is the gold standard model for conducting peripheral nerve experiments and has been well described in the literature [20]. An autograft model was evaluated as a nerve gap injury was used to evaluate a more severe nerve deficit than nerve transection, which can be repaired via primary repair. The autograft model entailed excision of a 10 mm segment of nerve, followed by repair using this 10 mm segment in a reversed fashion. The reverse orientation of the nerve fibers in the autograft serves to emulate variations in orientation of nerve fibers present when using isografts from expendable donor nerves in clinical practice.

Twenty- nine two-month old male Lewis rats were randomized to one of three experimental groups: 1) control with no nerve wrap 2) collagen matrix nerve wrap (Integra NeuraWrap) 3) hAM nerve wrap (Vivex Cygnus). The three groups consisted of 10, 9 and 10 animals, respectively, reaching 12-week endpoints. Control animals served to compare pain sensitivity, tissue repair, and locomotor changes to treatment groups. A 10 mm segment of the sciatic nerve was reversed 180 degrees and coapted with the proximal and distal nerve stumps. Adjunctive treatments were applied depending on the treatment group, with no additional wrap for the control. The hAM and collagen nerve wraps were applied to cover the entire autograft including both proximal and distal coaptation sites. hAM nerve wraps were applied with the epithelial side facing upwards and the stromal side in contact with the nerve.

Baseline behavioral testing was completed on all animals. This included CatWalk XT (CW) analysis and walking track analysis (WTA). Repeat testing was performed at 4, 8, and 12-week post-operative endpoints. Electrophysiological analysis was performed at 8, 10 and 12 weeks. At the 12-week endpoint, the animals were euthanized and perfused. Sciatic nerves, spinal cords, gastrocnemius muscles and tibialis anterior muscles were harvested and prepared for histological or macroscopic evaluation of axonal regeneration, nerve adhesions and muscle atrophy.

Experimental procedures were carried out in accordance with the provisions of the Animal Welfare Act (1966), and the criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health [21]. Experimentation was approved by the University of Miami Institutional Animal Care and Use Committee and complied with federal and state guidelines concerning the use of animals in research.

2.2. Sciatic nerve model surgical intervention

A dorsolateral gluteal muscle splitting incision was made, and the sciatic nerve was exposed from the sciatic notch to the distal trifurcation. The sciatic nerve was sharply transected with a straight micro-scissor approximately 1 cm and 2 cm distal to the sciatic notch, in order to resect a 1 cm segment of the sciatic nerve. The sciatic nerve was reversed 180 degrees and re-implanted across the defect, and the epineurium of the resected nerve segment was coapted with the proximal and distal stumps of the sciatic nerve in an end-to-end fashion with 9-0 Nylon sutures. Depending on the treatment group, the autografts were then wrapped with no wrap (control, Fig. 1a), hAM nerve wrap (Fig. 1b) or collagen matrix nerve wrap (Fig. 1c), which was secured to the epineurium of both the proximal and distal nerve stumps using sutures. Muscle, connective tissue, and the skin incision were then closed with 4-0 Vicryl sutures. Extended-release Buprenorphine and Meloxicam (1.2 mg/kg, 4 mg/kg) was administered to provide post-operative analgesia. The animal was placed on a homeo-thermic blanket system until recovery.

2.3. Behavioral analyses

Behavioral testing was completed at baseline prior to surgery, and at 4, 8, and 12 weeks on all animals to evaluate functional outcomes.

2.3.1. Walking track analysis

Walking Track Analysis (WTA) assesses recovery of sciatic nerve function after injury by examining hind-limb performance through footprints and the relationships between toes on the hind-limbs [22,23]. Animals were scored using the sciatic functional index (SFI), with 0 corresponding to normal function and −100 corresponding to complete dysfunction [22,23]. All animals were assessed prior to surgery for baseline SFI, and at 4, 8 and 12-week endpoints.

2.3.2. CatWalk XT analysis

CatWalk XT (Noldus, the Netherlands) (CW) behavioral testing is an established behavioral testing method in peripheral nerve research, and it is a highly sensitive tool to assess gait and locomotion [24]. The CW system is an automated gait analysis system used to assess motor function and coordination in rodent models. CW automated gait analysis overcomes limitations found in other methods of behavioral testing and is considered a more objective tool for evaluating functional outcomes. Results of CW behavioral testing have been shown to correlate with other behavioral methods used, including mechanical withdrawal thresholds.

Rats were first acclimatized to the CW System, then underwent conditioned locomotion along a runway. Subjects walked across a 1.3 m long black tunnel with an illuminated glass platform while a high-speed video camera was recording from below. The CW device tracked the foot placement and positioning of the rat's paws during conditioned walking along the runway and gathered information about various parameters of locomotion including stride length as well as limb couplings, paw print size, and print intensity/ weight distribution. Many gait-related parameters of mean stance duration and mean paw swing duration were reported and analyzed for each animal. The CW software, Illuminated Footprints Technology^{TM}, was used to capture actual footprints.

2.4. Histological analyses

Animals from each experimental group were sacrificed at 12-week endpoints. Following administration of Ketamine (40–100 mg/kg) and Xylazine (5–13 mg/kg), whole-body perfusion with 4% paraformaldehyde was performed to facilitate tissue preservation and histological study. Following perfusion, spinal cords, sciatic nerves, gastrocnemius

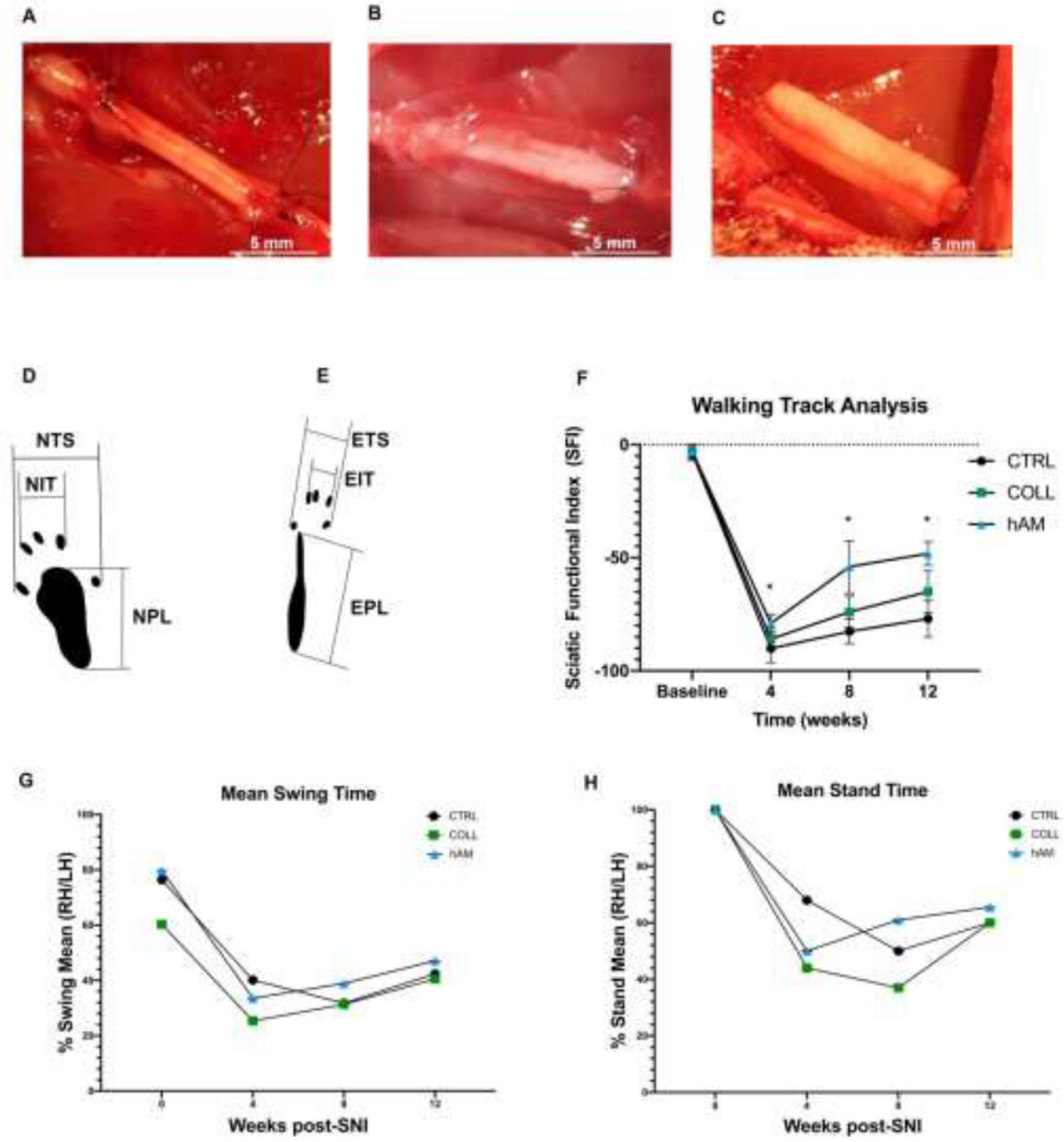

Fig. 1. Intra-operative photographs and functional evaluation: 1. Intraoperative photographs of sciatic nerve reverse autografts wrapped with no wrap (control) (1a), hAM nerve wrap (1b), or collagen nerve wrap (1c). Parameters used for Walking Track Analysis (WTA) and Sciatic Functional Index (SFI) included index print length (PL), toe spread (TS), and intermediary toe spread (IT) recorded on control as NPL, NTS, and NIT (1d) and experimental limbs as EPL, ETS, and EIT (1e). 1c. WTA of footprints and SFI demonstrated that hAM and collagen treated animals both had greater improvements in SFI compared to controls, with the hAM having the greatest improvement. There were significant differences between all groups at 8 and 12 weeks ($p < 0.05$). 1f. CatWalk XT automated gait analysis system was used for objective assessment of motor function and coordination. hAM-treated animals had a higher average mean swing time (1g) on the injured limb as well as higher mean stand speed (1h) at 8 weeks.

and tibialis anterior muscles contralateral and ipsilateral to the site of injury were harvested. The sciatic nerve reverse autograft (10 mm) and regions of the sciatic nerve adjacent to both coaptation sites (0.5 cm) were excised from the nerve (2 cm total). Harvested tissue samples were immersed in 4% paraformaldehyde overnight and were subsequently embedded in paraffin Nerves were sectioned transversely in 20 μm thickness, with 10 sections per slide and 2 slides per animal. A 3 cm section of spinal cord encompassing the L3-L6 motor pools was excised. Spinal cords were sectioned coronally with 10 μm thickness. There were 4 sections of spinal cord per slide, with 2 slides per animal.

2.4.1. Immunohistochemistry

Primary antibodies used for immunofluorescence included mouse monoclonal RT-97 (1:100, Developmental Hybridoma Studies Bank, Anti-Neurofilament 200 kDa (NF200)) and chicken anti-myelin P0 antibody. Secondary antibodies included Alexafluor-647, Alexafluor-594, and Alexafluor-488. Negative controls were processed simultaneously using blocking solution (5% goat serum). Sections were double-labeled with two different fluorophores and processed for immunohistochemistry using neurofilament heavy chain markers and myelin markers and Hoechst nuclear stains. The sections were pre-incubated with blocking solution (5% goat serum) at room temperature for 60 minutes (all primary and secondary antisera were diluted in this solution) and then incubated in a humid atmosphere with mouse anti-NF200 (1:100, Developmental Hybridoma Studies Bank, Anti-Neurofilament 200 kDa and chicken anti-myelin protein zero (Novus Biologicals) overnight at room temperature. Thereafter, the sections were rinsed in Sudan Black (0.05%), followed by PBS (1 × 5 min), ddH2O (2 × 5 min), and

PBS (2 × 5 min). Sections were then incubated with Alexafluor647-conjugated goat anti-mouse IgG and Alexafluor-594 conjugated goat anti-chicken IgG secondary antibodies (Jackson ImmunoResearch, PA, USA, dilution 1:1000) and Hoechst (1:500) for 3 h at room temperature, followed by rinsing in PBS (3 × 5 min) and rinsing in ddH2O (2 × 5 min). Sections were then mounted and coverslipped using Prolong Diamond mounting medium (Prolong Diamond Antifade 10). Fluorescently labeled tissues were viewed with a laser scanning confocal slide scanner microscope (Olympus VS120, Olympus, Center Valley, PA, USA).

2.4.2. Retrograde labeling

Cholera Toxin subunit B (CTB) was used for retrograde labeling of muscle-identified motor neurons. Intramuscular injections of CTB in target muscles can allow for characterization of the neuromuscular junction via quantification of the number of axons innervating the muscle at endpoints. Motor pools in rats were pre-labeled by intramuscular microinjection of retrogradely transported CTB, 1 week before conducting terminal experiments. Under aseptic conditions, the area of the hindlimb to be injected was exposed via a small incision to the hindlimb, and rats were injected unilaterally with 0.1% CTB (1.5–2 ul/site) using a Hamilton syringe. The skin incision was closed with absorbable 4-0 Vicryl sutures. A measured volume of CTB was loaded into the glass electrode by suction and is delivered to the muscle using slight positive pressure.

2.4.3. Histological evaluation of nerve adhesions: gomori trichrome staining

Twenty-micrometer-thick central transverse sections of the sciatic nerve and the surrounding neural bed were stained with the Gomori trichrome stain to evaluate areas of scarring surrounding the nerve.

2.5. Electrophysiology

Under anesthesia, the sciatic nerve was stimulated percutaneously through a pair of monopolar needle electrodes at the sciatic notch with single monophasic electrical pulses (20 μs, supramaximal intensity). Electromyographic (EMG) signals were recorded from gastrocnemius (GM) muscles. The compound muscle action potentials (CMAPs) were recorded by small needle electrodes placed in each muscle, amplified by × 100 or × 1000 (P511AC amplifiers, Grass) and band-pass filtered (3 Hz to 1 kHz). Digital sampling of the signals was made with a CED recording system (CED1401 Micro3) at 20 kHz and fed into Signal software. The onset latency and the peak-to-peak amplitude of each CMAP (M wave) were measured. During the experiments the animal body temperature was maintained by means of a thermostatic heating pad. Control values were recorded from the intact left hind limb.

2.6. Macroscopic evaluation

2.6.1. Adhesion scores

Macroscopic evaluation of adhesions of the sciatic nerve to surrounding muscles were evaluated at the 12-week endpoint during re-exposure of the sciatic nerves. The degree of nerve adhesions was assessed via the Petersen nerve adhesion grading scale, a numerical grading scheme for macroscopic evaluation of nerve adhesions as defined by Petersen et al [25]. Perineural adhesions with the surrounding muscles and nerve separability were classified into three different categories. Grade 1 is defined as no dissection or mild blunt dissection, grade 2 as some vigorous blunt dissection required, and grade 3 as sharp dissection required. The grading was performed by two investigators who were blinded to the experimental groups.

2.6.2. Gastrocnemius muscle weight ratios

The gastrocnemius muscles on both the control and experimental sides were resected and their wet weight was measured at 12 weeks. The ratio of experimental (ipsilateral to injured side) to control (contralateral to injured side) gastrocnemius muscle weights was assessed.

2.7. Quantitative and statistical analysis

The number of neurofilament antibody-positive axons in the sciatic nerve distal to the coaptation sites in control and experimental animals were counted by a blinded investigator using ImageJ software (National Institutes of Health). Sciatic nerve cross sections (20 μm, 20X) from approximately 0.5 cm distal to the most distal coaptation site of the nerve autograft were analyzed. Axon counts were performed in Image J and quantification was performed using the Grid Overlay tool and the Cell Counter tool from the Analysis menu of Image J. Systematic random sampling was used to evaluate alternating frames for a minimum of ten frames, after which mean axon counts for the sampling frames were obtained and multiplied by the area of the nerve cross-section to obtain the total axon count per nerve. Retrogradely-labelled neurons were also counted in Image J to generate total counts of CTB-labelled neurons throughout each spinal cord. A 3cm section of spinal cord encompassing the entry point of the motor neurons intro the spinal cord and the L3-L6 motor pools was evaluated. The perineural scarring area and the inner nerve area of Gomori trichrome-stained nerves was also measured in Image J, and the ratio of perineural scarring to inner nerve area was calculated.

The statistical significance of macroscopic evaluation, morphometric evaluation, functional evaluation (SFI and CW) and electrophysiological evaluation results were assessed using the one-way analysis of variance test (ANOVA) with Tukey HSD post-hoc testing or the Kruskal-Wallis nonparametric test. Assumptions of parametric analysis were confirmed prior to analysis. The Kolmogorov-Smirnov and Shapiro-Wilk tests using SPSS were performed to assess normality. Equal variance testing was performed using Bartlett's Test of Sphericity. All of the quantitative results were expressed as the mean (± SD). p <0.05 was considered to be statistically significant. Statistical analysis was performed using IBM SPSS statistical package version 26 (Armonk, New York).

3. Results

3.1. Behavioral Analysis

hAM and collagen treated animals both had greater improvements in SFI compared to controls, with the hAM having the greatest improvement (Fig. 1d, e, f). There were significant differences between all groups at 8 and 12 weeks ($p < 0.05$). At 4 weeks, SFI for hAM animals was significantly improved compared to collagen and control animals ($p < 0.05$), but there was no significant difference between collagen and control animals (Fig. 1f).

CatWalk analysis demonstrated that hAM treated animals had in a higher average mean swing time (Fig. 1g) on the injured limb as well as an improved mean stand time (Fig. 1h) at 8 and 12 weeks, indicating a greater functional improvement in hAM outcomes relative to collagen and controls; however, these differences were not significant.

3.2. Histological Analysis and Macroscopic Findings

hAM-treated nerves had significantly higher numbers of axons compared to control animals ($p < 0.05$) (Fig. 2a, b). hAM-treated nerves had significantly fewer adhesions at 12 weeks post-injury compared to control and collagen treated nerves ($p < 0.05$) (Fig. 3a, b, c). hAM-treated nerves also had significantly less perineural fibrosis at 12 weeks post-injury compared to collagen and controls ($p < 0.05$) (Fig. 3d). Retrograde labeling demonstrated that the ratio of experimental to uninjured retrograde labeled motor neurons in the amniotic membrane group was significantly greater than in the collagen and control groups ($p < 0.05$) (Fig. 4a, b). It was also significantly greater in the collagen group compared to controls ($p < 0.05$).

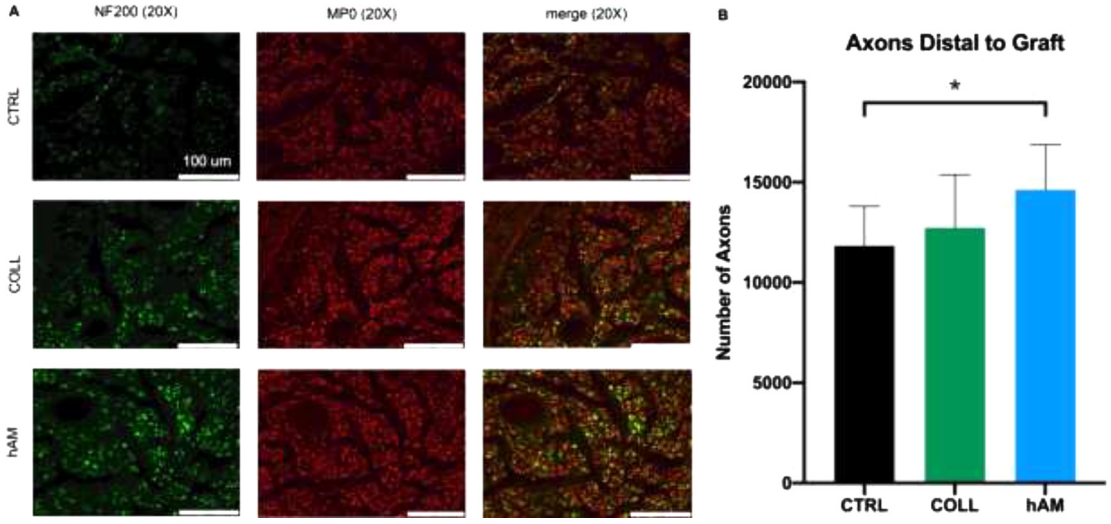

Fig. 2. Intraoperative nerve wrap photographs and histological evaluation of sciatic nerve cross sections: 2a. Sciatic nerve cross sections (20 μm, 20X) analyzed approximately 0.5 cm distal to coaptation site. Axons were fluorescently labeled with Anti-Neurofilament 200 kDa primary antibody and Goat Anti-rabbit Alexa Flour 488 secondary antibody and myelin sheaths were labeled with Anti-Myelin Protein Zero primary antibody and Goat Anti-chicken Alexa Flour 594 secondary antibody. The merged panel depicts multichannel overlays. Figure 2b. Immunohistochemical analysis demonstrated that human amniotic membrane-treated animals had significantly higher numbers of axons compared to controls, while collagen-treated animals did not ($p < 0.05$, $p > 0.05$). There was no significant difference between collagen and human amniotic membrane groups ($p > 0.05$).

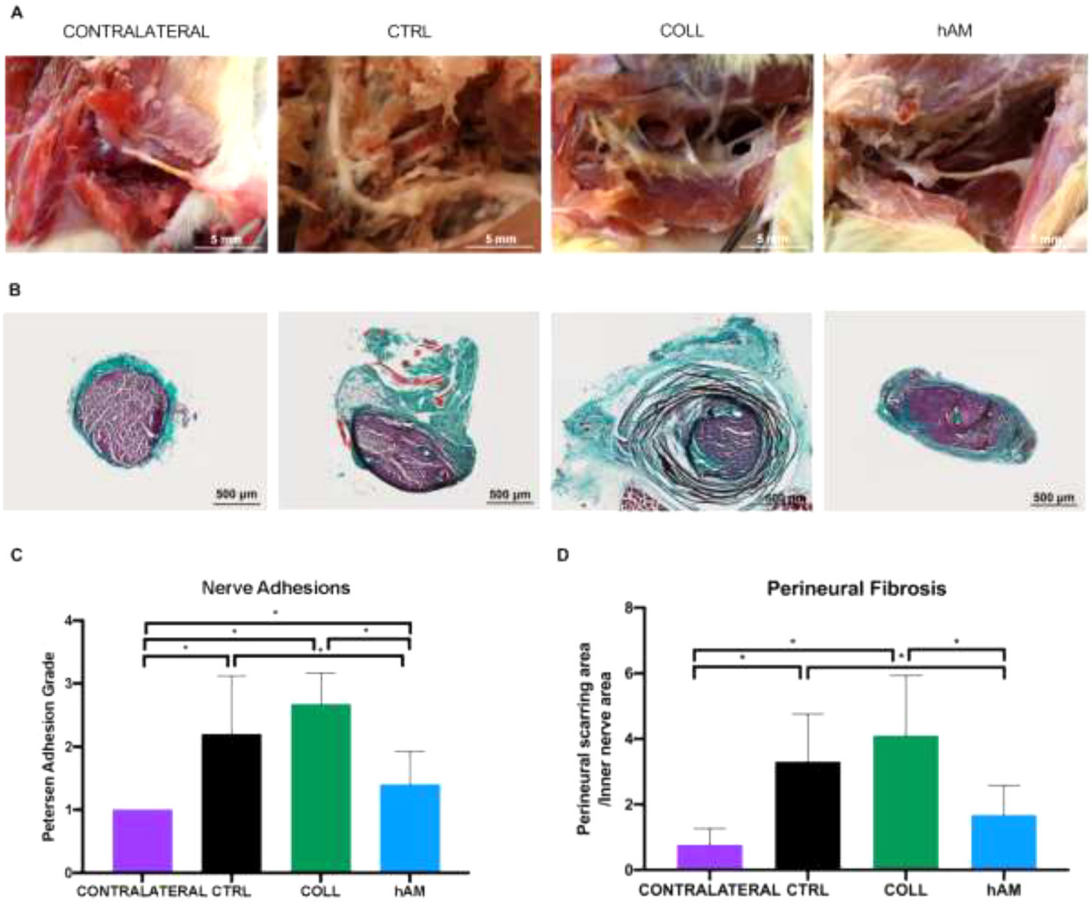

Fig. 3. Evaluation of adhesions and fibrosis: 3a. Macroscopic view of sciatic nerve following re-exposure at 12 weeks. Control injured nerves were often adhered to muscle, as were collagen-treated nerves, while hAM nerves exhibited a greater degree of liberation from the surrounding muscle and less scarring and adhesions. 3b. Examples of histologic sections taken from each group (20 μm, 5X) taken from the distal coaptation site of the experimental sciatic nerve were stained with the Gomori Trichrome Stain in order to evaluate perineural fibrosis (nuclei: purple, collagen: teal). 3c. hAM samples had significantly fewer adhesions surrounding the nerve at 12 weeks post-SNI compared to collagen and control nerves. 3d. Quantification of perineural fibrosis: The ratio represents the relative area of perineural scarring to the area within the epineurium. Control and collagen nerves had significantly more perineural scarring than hAM-treated nerves ($p < 0.05$).

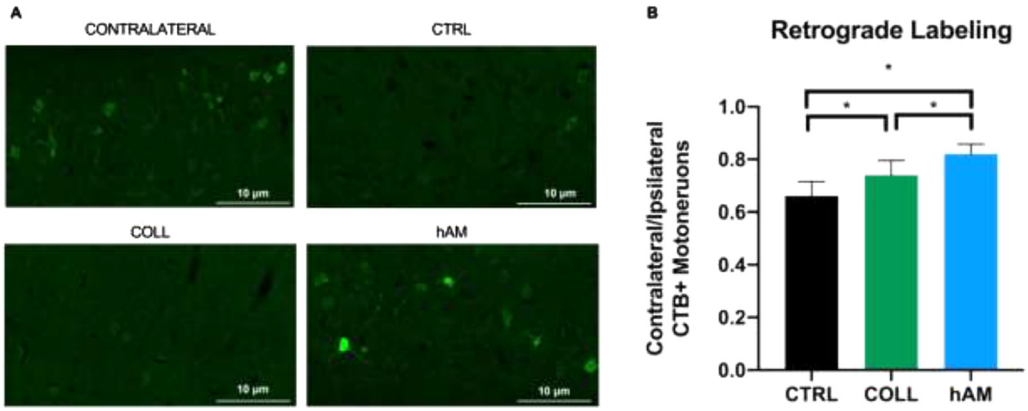

Fig. 4. CTB retrograde labeling: 4a. Examples of histologic sections taken from each treatment group (20 μm, 15X) taken of the lumbosacral region of the spinal cord demonstrating retrograde-labeled motor neurons from the ipsilateral (experimental or control) or contralateral (control) hind-limb following micro-injection of CTB neuronal tracer into the gastrocnemius. 4b. Retrograde labeling demonstrated that significantly greater numbers of motoneurons had regenerated axons in the human amniotic membrane group compared to the control and collagen groups ($p > 0.05$). There were also significantly greater numbers of motoneurons in the collagen compared to the control group ($p > 0.05$).

3.3. Gastrocnemius muscle weight ratios and electrophysiological findings

The ratio of experimental to control gastrocnemius weights was significantly greater in hAM compared to control samples ($p < 0.05$) (Fig. 5a, b). The normalized CMAP indicated more efficient improvement with hAM (Fig. 5c-f). The normalized CMAP of hAM animals ($n = 6$) was significantly improved compared to collagen ($n = 4$) and controls ($n = 5$) at 10 weeks ($p < 0.05$).

4. Discussion

Over twenty million Americans are estimated to suffer from peripheral nerve injuries [26]. Surgical repair is commonly required in cases of traumatic injury. Additionally, nerve coaptations are performed in non-acute/traumatic settings, as is the case for nerve transfers [27]. Autologous nerve grafting is the preferred method for long gap nerve injuries where primary repair is not possible [26]. Nerve wraps can be employed at the time of neurorrhaphy, to protect the repair and potentially minimize adhesion formation [10,28].

Nerve wraps composed of biomaterials such as collagen and hAM have been shown to reduce scarring and fibrosis of injured peripheral nerves [9]. Various studies have reported that collagen nerve wraps may reduce adhesions and neuroma formation following peripheral nerve repair [9, 11,12]. hAM has also been shown to reduce adhesions, and has been shown to have anti-fibrotic, anti-scarring, pro-regenerative, and unique immunomodulatory properties, resulting in neuroprotective effects and enhanced peripheral nerve regeneration in the setting of peripheral nerve injury [9,10,13,14,15].

Although various clinical and preclinical studies have reported positive outcomes of hAM and collagen nerve wraps, indicating they have the potential to enhance peripheral nerve regeneration, a direct comparison has not been investigated [9]. In this preclinical randomized control trial, we utilized a rat sciatic nerve reverse autograft model to compare the efficacy of hAM and collagen nerve wraps for peripheral nerve regeneration via functional, electrophysiological, macroscopic, immunochemical, and histological evaluation. Our study demonstrated that hAM-treated nerves had significantly less perineural fibrosis and nerve adhesions compared to control and collagen-treated nerves. Interestingly, there was no significant difference in perineural fibrosis between human amniotic membrane-treated nerves and uninjured contralateral nerves. This finding highlights the ability of hAM to reduce the formation of adhesions at the site of a healing nerve injury. In contrast, collagen-treated nerves exhibited the greatest amounts of perineural fibrosis and adhesions. These findings correspond to the study by Lemke et al., who observed that transplantation of human amnion prevents recurring adhesion and ameliorates fibrosis in a rat model of sciatic nerve scarring [10]. hAM MSCs are characterized by anti-inflammatory properties that allow them to overcome common limitations of other types of stem cells such as inflammatio-mediated rejection and poor differentiation and proliferation. hAM is an inexpensive source of embryo-derived stem cells for clinical applications in regenerative medicine that is isolated in a non-invasive manner. hAM-derived cells have been shown to exhibit multi-differentiation potential [29]. It contains human amniotic mesenchymal stem cells (hAMSCs) which have unique properties including inflammation suppression, angiogenesis promotion, oxidative stress inhibition, neurogenesis induction and differentiation of progenitor cells into neurons, neuroprotection, metallomatrix proteinase regulation, and remyelination stimulation, which are of therapeutic value for prevention of peripheral nerve injury and enhancement of peripheral nerve regeneration [30]. Neurotrophic and angiogenic factors produced by hAMSCs have been shown to produce a supportive microenvironment for neural regeneration supplementing tissues with growth factors essential for regeneration and neovascularization [31,32]. While the exact role of hAMSCs (structural vs. immunomodulatory) continues to be defined, it has been demonstrated that hAMSCs stimulate tissue repair via release of trophic factors, and they produce proteins and cytokines that stimulate tissue regeneration with immunomodulatory effects. hAMSCs are hypothesized to not only stimulate tissue regeneration but also form extracellular matrix components, enhance angiogenesis, inhibit scarring and fibrosis, and mitigate the inflammatory response and inhibit oxidative stress [15]. In our study, use of hAM nerve wraps results in superior nerve regeneration and functional recovery compared to collagen nerve wraps. Immunohistochemical analysis demonstrated that hAM-treated animals had significantly greater regeneration of axons compared to controls. This relationship was not demonstrated in the collagen-treated animals. Direct comparison between collagen and human amniotic membrane groups showed no significant difference. This is likely due to the protective nature of a nerve wrap in itself, independent of the biochemical makeup, and the ability of the wrap to create an optimal microenvironment for axon regeneration by protecting the epineurium from fibrotic adhesions and improving nerve gliding [33].

A significant element of the efficacy of hAMSCs is mediated by paracrine effects and secreted trophic factors. hAMSCs are promising for improving outcomes of peripheral nerve regeneration, secreting and recruiting growth factors such as GDNF and VEGF, and ultimately resulting in improved functional outcomes following peripheral nerve repair [34]. Growth factors such as VEGF have been shown to result in improved angiogenesis in the setting of peripheral nerve injury [31]. We

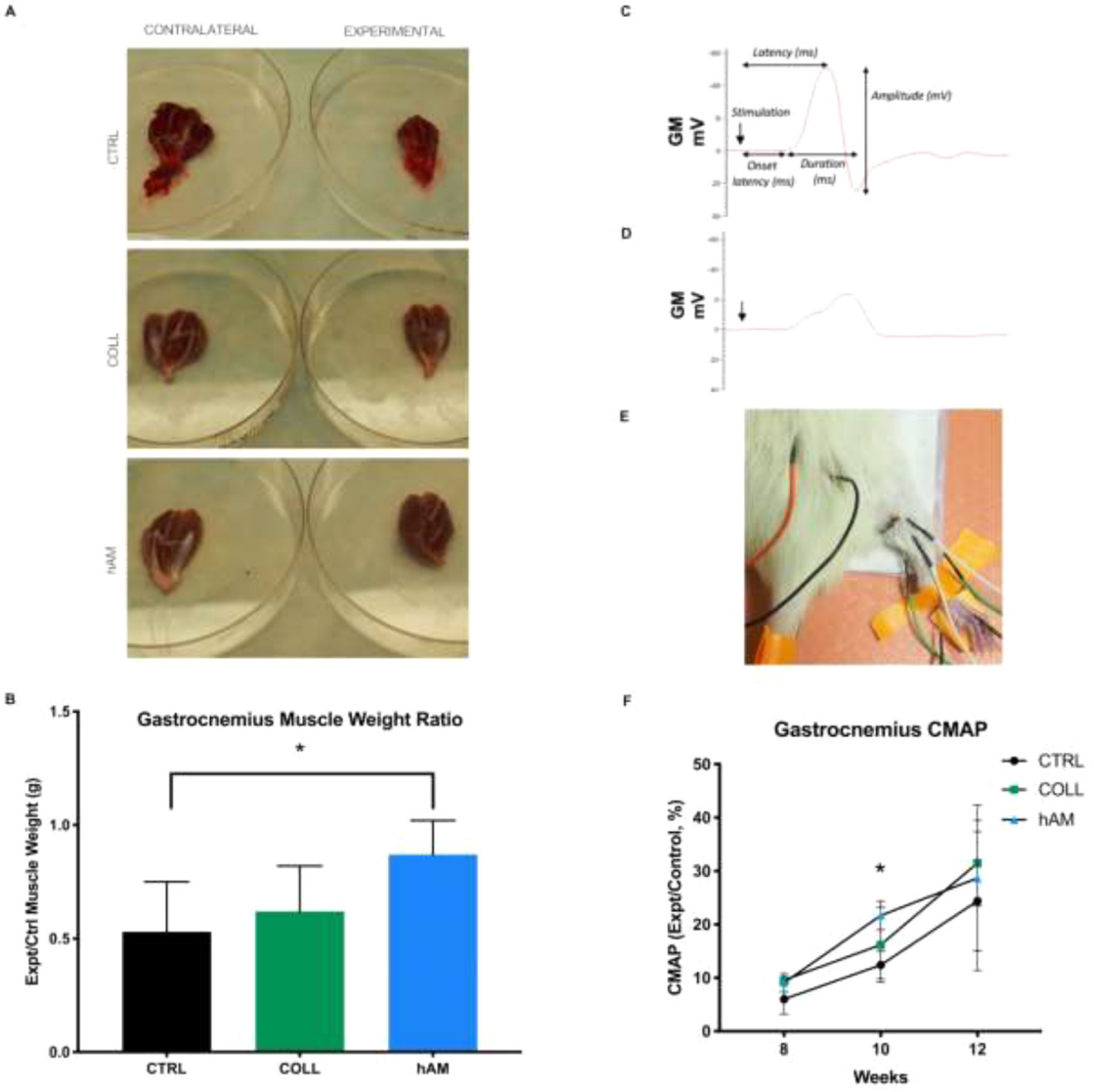

Fig. 5. Gastrocnemius muscle weight analysis and electrophysiological analysis: 5a. Gastrocnemius muscle resection at 12-week endpoint. 5b. The ratio of experimental to control gastrocnemius weights was significantly greater in hAM-treated animals compared to control animals at 12 weeks post-SNI. 5c. Electromyographic signals were recorded from the gastrocnemius muscles. The peak-to-peak amplitude of each CMAP (M wave) was measured. CMAP values were recorded from the uninjured contralateral limb (5d) and the injured ipsilateral limb (5e). The ratio of experimental to control CMAP was evaluated and was significantly greater in the hAM group at 10 weeks compared to collagen and controls ($p < 0.05$).

evaluated numbers of motor neurons between the lumbar 3-6 vertebral bodies via retrograde labeling with Cholera toxin B neuronal tracer via the gastrocnemius to evaluate successful reinnervation of motor endplates. Retrograde labeling demonstrated that the ratio of experimental to uninjured retrograde labeled motor neurons in the amniotic membrane group was significantly greater than in the collagen and control groups, corroborating the nerve fiber quantification results at the injury site. It was also significantly greater in the collagen group compared to controls. The ratio of experimental to control gastrocnemius weights was significantly greater in the hAM-treated group compared to the control groups. Improved gastrocnemius muscle weight findings as well as improved axonal regeneration findings indicate that a greater number of axons successfully reinnervated motor end plates in hAM-treated animals compared to collagen treated animals and controls. These findings are in line with studies have shown that hAMSCs promote neurological recovery and subsequent motor recovery in rats after central and peripheral nervous system injuries [30]. The hypothesized mechanism of action is that hAMSC-related neuroprotection and nerve repair is mediated by inhibition of inflammation and apoptosis, increasing neurotrophic factors expression, promoting neurogenesis and angiogenesis, and induction of differentiation of haMSC progenitor cells into neurons [10,13,16,17,35,36,37]. hAMSCs may improve peripheral nerve regeneration by enhancing neurogenesis, neuroprotection, and remyelination via increasing the expression of neurotrophic factors including nerve growth factor, ciliary neurotrophic factor and brain-derived neurotrophic factor [38]. Neurotrophic factors inhibit initiation of programmed cell death in neurons and enhance survival, development, and function of neurons [30,37,39,40]. The release of neurotrophic factors are suggested to directly improve functional outcomes [10,13,31,35]. hAM has also been shown to upregulate local Schwann cell proliferation and increase neurite outgrowth from DRGs [16,17].

Histological and gross muscle findings corresponded with functional and electrophysiological outcomes. WTA was performed to evaluate functional recovery using the SFI. The SFI is considered the most important parameter to assess functional recovery in rats following sciatic nerve injury. hAM and collagen-treated animals both had greater

improvements in SFI compared to controls, with the human amniotic membrane having the greatest improvement. Both hAM and collagen nerve wrap-treated animals had significant improvements in SFI compared to controls at 8 and 12 weeks. However, only hAM-treated animals had significant improvements in SFI compared to controls at 4 weeks, whereas there was no significant difference between collagen and control animals. This may be indicative of hAM having a neuroprotective effect at the time of nerve injury and mitigating neurotoxicity, contributing to more rapid nerve regeneration due to its regenerative properties, or a combination of these two effects. Collagen nerve wraps may have improved nerve regeneration outcomes at later end points due to the protective nature of the nerve wrap, but this may not have occurred as quickly and effectively as compared to hAM nerve wraps as collagen lacks neuroprotective and pro-regenerative properties. WTA analysis demonstrated that the hAM-treated group resulted in improved outcomes starting from an early time-point. The CW automated gait analysis system was used in addition to WTA to assess motor function and coordination. haM-treated animals had a higher average mean stand time on the injured limb as well as an improved mean swing time compared to collagen-treated animals and controls, meaning their gait pattern was more regular at the final endpoints, though these differences were not significant. Electrophysiological analysis demonstrated that the normalized compound muscle action potential of hAM-treated animals was significantly improved compared to collagen-treated animals and controls at 10 weeks. These findings support the capability of hAM to improve outcomes, particularly at earlier time points. This indicates that hAM may enhance the nerve regeneration process at an early time frame critical to nerve regeneration, leading to overall improved outcomes. Improvements did tend to taper off at later time points, which may be reflective of the combination of a decrease in the nerve regeneration process and the degradation of the amniotic membrane.

4.1. Limitations and future directions

Our study is limited by sample size and a single endpoint at 12 weeks, which did not allow for serial evaluation over time for histological evaluation. However, SFI did allow for serial evaluation of functional recovery over time. Future directions include the development of strategies for improvement of the molecular biostability and durability of hAM. Enzymatic degradation of the hAM matrix is considered a shortcoming of hAM after surgical transplantation [41]. However, the actual rate of degradation or the length of time hAM growth factors and stem cells exert a neurotrophic effect has not been clearly defined. Studies from the Ophthalmology literature have shown that hAM does not degrade for up to 6 weeks after being applied for corneal ulcer repair [42]. Studies from the vascular literature have shown that hAM conduits for vascular repair remain for at least 24 weeks [43]. Research elucidating the degradation rate of hAM and evaluating stem cell fates in the setting of peripheral nerve repair is warranted [42]. The goal of our study was not to quantify the timeline of hAM degradation; however, it is worth noting that the hAM was noted to be present at 12 weeks when animals were euthanized and nerves harvested, appearing as a thin layer integrated around the epineurium.

5. Conclusions

Application of both hAM and collagen nerve wraps in a rodent sciatic nerve reverse autograft model resulted in improvements in functional and histological outcomes compared to controls, with the greatest improvements seen in the group treated with hAM nerve wraps. Our findings demonstrate that the use of hAM nerve wraps following peripheral nerve injury and repair reduces adhesions and fibrosis, and that hAM may have a neurotrophic effect that results in improved nerve regeneration and functional recovery in a rat sciatic nerve reverse autograft model compared to controls. The anti-fibrotic, anti-inflammatory and pro-regenerative properties of hAM and its ability to improve peripheral nerve regeneration early on in the nerve regeneration process make it a promising biomaterial for clinical applications in peripheral nerve repair.

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Data statement

Data is available upon request.

Declaration of Competing Interest

The authors have no financial interests in any of the products mentioned in this discussion. None of the authors or any member of his or her immediate family has funding or commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

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