

This is a provisional PDF only. Copyedited and fully formatted version will be made available soon.



ISSN: 0015-5659

e-ISSN: 1644-3284

Biodegradable nanofiber coated human umbilical cord as nerve scaffold for sciatic nerve regeneration in albino Wistar rats

Authors: Balasundari Ramesh, Jaikanth Chandrasekaran, Kotturathu Mammen Cherian, Adegbenro Omotuyi John Fakoya

DOI: 10.5603/FM.a2023.0022

Article type: Original article

Submitted: 2023-01-03

Accepted: 2023-03-12

Published online: 2023-03-23

This article has been peer reviewed and published immediately upon acceptance. It is an open access article, which means that it can be downloaded, printed, and distributed freely, provided the work is properly cited.

Articles in "Folia Morphologica" are listed in PubMed.

Biodegradable nanofiber coated human umbilical cord as nerve scaffold for sciatic nerve regeneration in albino Wistar rats

Balasundari Ramesh et al., Nerve scaffold for sciatic nerve regeneration

Balasundari Ramesh¹, Jaikanth Chandrasekaran¹, Kotturathu Mammen Cherian¹, Adegbenro Omotuyi John Fakoya²

¹Frontier Lifeline Pvt Ltd, R3OC Ambattur Industrial Estate Road, Chennai, India

²Louisiana State University, Health Sciences Center, Shreveport, United States

Address for correspondence: Prof. Adegbenro Omotuyi John Fakoya, Department of Cellular Biology and Anatomy, Louisiana State University, Health Sciences Center, 1501 Kings Highway P.O. Box 33932, 71103 Shreveport, United States, e-mail: adegbenro.fakoya@lsuhs.edu

ABSTRACT

Background: Human umbilical cord (hUC) is encompassed by a mucoid connective tissue called Wharton's jelly (WJ), made of hyaluronic acid, collagen, and stromal cells to support the blood vessels of hUC. This study was aimed to determine the *in vitro* neuronal differentiation of Wharton's jelly-derived Mesenchymal stem cells (WJMSCs), and *in vivo* axonal regeneration potential of nanofiber coated human Wharton's jelly as a neuronal graft after sciatic nerve injury in immunosuppressed Albino Wistar rats.

Materials and methods: WJMSCs could be differentiated to neuron-like cells by inducing with neuronically supplementing media. The test animal's axotomized nerves were implanted with trimmed human umbilical cord devoid of vascularity and nanocoated with electro-spun PLLA nanofibers. The control animals were bridged with native sciatic nerve reversed and sutured. Post-surgical functional recovery was studied by walking track, pinprick, muscle weight, and sweating quantification. At the end of the 4th week, the animals were euthanized, and Magneto Neuro graph (MNG) was performed. The explanted grafts were quantified by immunohistochemistry for immuno-rejection, neural scarring, neural adhesion axon regeneration, fiber diameter, myelin thickness, and g-ratio. The sciatic function index (SFI) values were similar by walking track analysis for both the test and control groups.

Results: The animals had functional and sensation recovery by the end of two weeks. No mortality, signs of inflammation, and acute immune rejection were observed post-surgery.

Conclusions: The hUCWJ devoid of vascular elements can be a perfect peripheral nerve graft, and we hypothesize that the cryopreserved hUC could be an ideal resource for axonal regeneration in the future.

Key words: umbilical cord, Wharton's jelly, mesenchymal stem cells, nanofiber, sciatic nerve, axonal regeneration

INTRODUCTION

Traumatic peripheral nerve injury is a global problem and can cause devastating functional disabilities [11]. Several hundred thousand cases of peripheral nerve injury occur each year, results from stretch-related injuries, lacerations, or compressive trauma. The affected limb displays painful neuropathy symptoms, such as hyperalgesia, pain-related gait, and swelling. Ascending spinal pathways subsequently distribute nociceptive pain to multiple cortical, limbic structures and hypothalamus [15]. Current treatment modalities for peripheral nerve injuries (PNI) involve end-to-end suturing of uninjured nerve ends for minor injuries and the use of autologous nerve grafts and neural prosthesis for significant damages. The application of nerve grafts for peripheral nerve repair (PNR) is associated with donor site morbidity, the need for multiple surgeries, limited tissue availability, and inadequate functional reinnervation [28]. Allografts and cell suspension from the fetal Central nervous system (CNS) as the most effective neuro graft and have been substantially studied for their ability to develop and integrate within the host organism. However, ethical, social, and political issues act as a barrier to the application of fetal tissues [3,13].

The unique morphologic properties of the human umbilical cord make them productive and the alternate source for Mesenchymal stem cells (UC-MSCs), primarily as compared with umbilical cord blood [4,16]. The UC-MSCs secretes different cytokines, chemokines, and growth factors known neurogenic cytokines, such as Neurotrophin 3 (NTF3), Endothelial growth factor (EGF), and Midkine (MDK), may be a potential MSC source for neural stem cell therapies. The extracellular matrix of human umbilical cords is rich in collagen IV and V, hyaluronan, and sulfated glycosaminoglycans (GAGs) [14,21].

In the present basic research, we investigated the *in vitro* differentiation of hUC-MSCs to Neuron-like cells. The differentiated cells were verified for positive expression of neuronal markers by a reverse transcriptase-polymerase chain reaction and immunocytochemistry.

Furthermore, an attempt was made to use human umbilical cord devoid of blood vessels as a neural graft to replace axotomized sciatic nerve in Albino Wistar rats to restore impaired motor or sensory functions.

MATERIALS AND METHODS

The study was conducted after proper approval from various committees, including the Animal ethical committee and the stem cell ethics committee (IC-SCR). All the animals involved in this research were treated humanely, and experiments were conducted as per the guidelines of the Committees for the purpose of Control and Supervision of Experiments on Animals (CPCSEA).

The animals were purchased from Tamil Nadu Veterinary and Animal Science University. The experimental grafts were recovered from animals after euthanasia was done by certified Veterinary technician. The euthanasia was done by using a pre-euthanasia anesthetic (Telazol or PreMix 0.5ml/10 pounds) to render the animal unconscious before injecting the euthanasian drug (Sodium Pentobarbital 117mg/pound). The control animals involved in the research were maintained till natural death in the small animal facility in Frontier Mediville. There were no criteria needed for euthanizing animals prior to the planned end of the experiment.

For the collection of the amniotic membrane and cord blood, a written informed consent was administered to the patients or their parents. The patients were prescreened for HBsAg, HIV I and II, HCV, CMV, and Venereal diseases. The study was conducted after a proper approval from various committees, which includes Animal ethical committee and stem cell ethics committee (IC-SCR)

In vitro studies: neuronic differentiation of Wharton's jelly-derived mesenchymal stem cells (WJMSCS)

Derivation of PLLA Nanofibers: PLLA nanofibers were derived by dissolving PLLA pellets in 1,1,1,3,3,3 hexafluoroisopropanol (HFIP, Sigma Aldrich 105228) and electrospun at a flow rate of 12–14 μ l/min, with an applied voltage of 10–18 kV and 15 cm from a 4.5% (w/v) polymer solution [26]. The nanofibers can be directly coated on cell culture plates for *in vitro* experience and human umbilical cords (hUCs) prior to *in vivo* experiments.

The hUCs were collected in Hanks balanced salt solution (HBSS – Himedia TS1003) with Antibiotic solution (Himedia A002A) from C-Section delivery and transported to the laboratory with chilled packs within 6 hours. The hUCs were washed well with normal saline (0.9% W/V) and Antibiotics to remove the residual blood from the veins and artery. Further, the

hUCs were dissected free from vascularity. The dissected tissues, approximately 50 mg were minced using sterile method into small fragments 1-2 mm. The minced tissue was also digested with 0.5 mg/ml collagenase (Sigma, C9722) and 0.2 mg/ml hyaluronidase (Sigma H3506) for 2 hours at 37°C. After enzymatic digestion, Cold Dulbecco's Phosphate buffered saline (DPBS, Himedia-TS1006) was added to cease the enzymatic activity. Further, the digested WJ was washed by centrifuging twice with DPBS at 1500 rpm. The pellet along tissue debris was plated in the PLLA nanofiber coated plates with Dulbecco's modified eagle's media (DMEM with 4500 mg/ml glucose) and 2 mM l-glutamine (GIBCO-119950), supplemented with 10% FBS (GIBCO- 26140079). After 48 hours, the floating cells and debris were removed. The 2/3 confluent flask with adherent fibroblast cells was passaged using TrypLE Select (GIBCO). The Passage 2 cells were characterized using immunocytochemistry and flow cytometry [24]

Characterisation of Wharton's jelly cells

Immunocytochemistry: The cells were fixed with 4% paraformaldehyde in PBS pH 7.4 for 15min at room temperature, followed by permeabilization with 0.25% Triton X and were incubated for 1 h with primary antibodies – Anti CD73 (Abcam ab54217), Anti CD90 (Abcam ab23894), Anti CD (Abcam-ab44967), Anti CD44 (Abcam- ab9124), Anti CD34 (Abcam-ab8536), Anti CD45 (Abcam-ab40763). The primary monoclonal antibodies were added to the six-well culture plates as 1/100 dilution in 5% Bovine Serum albumin (Himedia – TC 194). After that, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and incubated with Fluorescein isothiocyanate (FITC- Abcam ab5765) or Phycoerythrin (PE-Abcam ab97024) conjugated secondary antibody at room temperature for 1h. The nucleus was stained using 4',6-diamidino-2-phenylindole 300nm in Distilled water as per manufactures protocol (DAPI, Sigma D9542). The immune-stained cells were observed under fluorescent microscope (Olympus), and images were captured.

Flow cytometry: The cultured WJ-MSCs were detached with 0.25 % trypsin and 0.2 % EDTA, further blocked with 1% Bovine serum albumin in Dulbecco's phosphate-buffered saline (DPBS) and incubated with Abcam primary antibody (20 µgs/ml in 1% BSA) at 4 °C overnight. Cells were washed twice with DPBS and incubated with the fluorophore (FITC or PE) conjugated secondary antibody at room temperature for 30 min. The cells were fixed with 2% paraformaldehyde and approximately 10⁶ cells were subjected to flow cytometry by FACS Calibur (Becton- Dickinson) [24,25]

Induction of WJ-MSCS to neuronic differentiation

The WJ-MSCs at cell culture passage five were used for differentiation experiments. Briefly, WJ-MSCs were induced to neuronal lineage differentiation by serum-free Neurocult media (Stem cell technologies). Control cultures were maintained in DMEM media supplemented with 10% FBS. The cells were replenished with new medium every alternate day and cultured for three weeks in the medium. The cultures were maintained in triplicates, and significant change in cell morphology was noted. At the end of the 3rd week, the cells were fixed for Immunocytochemistry.[7]

In vivo small animal experiment

Surgical procedure: Sciatic nerve injury and graft implantation of biological nerve graft were conducted as described earlier methods [27,34]. Albino Wistar Rats 12 Nos male (6 Nos for umbilical grafts + 6 Nos for control autografts) approximately weighing 200 ± 20 gms were used for axonal regeneration studies. Wistar rats were anesthetized intraperitoneally by using Ketamin (75 mg/kg) and Xylazine (20 mg/kg). The left hind limb sciatic nerve was exposed through a gluteal muscle incision, and about 1 cm sciatic nerve was transected at the mid-thigh level. The umbilical cord graft was trimmed with stereomicroscope following sterile protocols to remove the blood vessels and to match the size (length and width) of rat sciatic nerves. The test group rat axotomized nerve was bridged by suturing with human umbilical cord. These animals were administered with hydrocortisone (50 mg/kg) to prevent immune rejection as human umbilical cords were transplanted in rats. The control group axotomized sciatic nerve was reversed and sutured. Behavior analysis was performed every week post-surgery to study the functional recovery of test and control rats by walking track analysis, pin-prick test, muscle weight, and sweating quantification. At the end of the 4th week, the animals were euthanized, and Magneto neuro graph (MNG) was performed in the umbilical cord grafted sciatic nerve, autografted sciatic nerve, and control native sciatic nerve from right hind limb. The explanted grafts were quantified by immunohistochemistry for immune-rejection, neural scarring, neural adhesion, axon regeneration, fiber diameter, myelin thickness, and g-ratio [8]

Functional tests were performed before the injury, immediately after transplantation, and after that weekly for 12 weeks after transplantation. Locomotor activity was evaluated to assess the animal's locomotor function using the walking track analysis as described below.

Walking track analysis

The most commonly used method of evaluation of the function of target organs after nerve injury in the rat is walking track analysis. [2, 10] The sciatic function index (SFI), is

calculated using three measures of hind leg footprints acquired by walking track analysis: the 1-5 toe spread (TS), the 2-4 toe spread (ITS), and the print length (PL). The SFI was measured at a weekly interval between the 1st and 8th postoperative weeks. Triple measures were performed for each print and represented statistically. SFI=0 was considered normal, whereas SFI=100 was considered to indicate full damage. N is the values for normal rat, and E is for experimental rat.

$$SFI = -38.3 \times \left(\frac{EPL - NPL}{NPL} \right) + 109.5 \times \left(\frac{ETS - NTS}{NTS} \right) + 13.3 \times \left(\frac{EIT - NIT}{NIT} \right) - 8.8$$

Pin-prick test (PP)

The pin-prick test (PP) was used for sensitivity repair recovery. The test consisted of the pin-prick with a forceps of the animal-operated hind limb to evaluate the sensory recovery. The animal was pin-pricked from the toes to the knee until limb withdrawal was observed. Sensory recovery was graded on a scale from 0 to 3. Where grade 0 - represent the lack of response to pain stimulus, grade 1- represents the limb withdrawal on stimuli above the ankle, grade II – withdrawal below the ankle, in the heel region, and grade III – limb withdrawal after the application of stimuli in the metatarsal region.

Toe spread test

Toe spread test was used for motor recovery evaluation. It is because the non-operated, normal rat extends and spreads the toes when it is hung by the tail. As in sensory recovery, motor recovery was graded on a scale from 0 to 3, where grade 0 – digital movement absence, grade I – any movement, grade II – toe spread, and grade III – toe extension and abduction.

Sweating quantification

The sweating of paws reflects autonomic nerve activity. Reinnervation of Albino Wistar rat's hind paws sweat glands after peripheral nerve injury can be quantified by counting active sweat glands after induction by Pilocarpine.

The Albino Wistar rats were anesthetized with ketamine and xylazine as above. The implanted left hind paw was cleaned with distilled water and dried using nitrogen gas, and pilocarpine (80 mg/kg) was administered through I.P route. The sweat droplets were collected for 2-3 min after injection. After collection, micropipettes were centrifuged for oil and sweat separation, and the aqueous layer was quantified to measure the sweat volume.

Magento neruography (MNG)

MNG endpoint measurements were carried out 4, 8, and 12 weeks following the ex vivo MNG technique following the protocol described by Cudlip et al., 2002. The animals were anesthetized with Urethane i.p. 12.5% (1ml/100gram). The sciatic nerve was exposed from its origin in the lumbar spine to distal to the trifurcation at knee level to acquire a segment of maximum length. To prevent axon leakage, the proximal part and the three branches of the sciatic nerve were ligated with a 6-0 suture and were transected proximally and distally to the knots, respectively. The MNG recording chamber was filled with Ringers Lactate buffer containing glucose, 1 g/L, at 21 ± 0.1 °C. The sciatic nerve was guided through the recording sensor coils and the stimulation cuff and stretched to 'in vivo' length by clamping the two sutures in the recording chamber. The distal nerve end was stimulated with a biphasic constant current pulse of 50 μ s delivered by two separate stimulus units (Digitimer DS3), connected in parallel. To guarantee supramaximal stimulation, the stimulator was finally set to 1.4 times the strength of the lowest current that produced a maximal signal. Every time the stimulation cuff changed position; this stimulus strength was redetermined. Both the right (Control- Normal nerve) and left (Test 6 Nos for Umbilical cord and 6 for native nerve graft surgery) sciatic nerve were measured. In the recording setting of the right nerve, the stimulation cuff cathode was positioned 10 mm distal to the suture line. Sensor 1 and sensor two were placed 4 mm and 14 mm proximal to the suture line, respectively. Only axons with fibers regenerating across the lesion were stimulated and recorded by applying distal stimulation and proximal recording. For the contralateral (unoperated) nerve, similar recording settings were used, and an imaginary suture line was created at the same distance from the distal nerve end as present in the repaired nerve. [29]

Muscle weight

After explanting the sciatic nerves for MNG measurement, the gastrocnemius muscles were simultaneously dissected and detached from their origin, insertion and weighed immediately. Muscle weight was expressed as the ratio of the left (operated) over the right (control) muscle weight.

Histological and immunohistochemical studies

At sacrifice four weeks later, the nerves were histologically evaluated for traumatic neuroma formation, Wallerian degeneration, perineural scar formation, and morphometric analysis. The distal sections were analyzed for the differences in total fascicular area, myelinated

fibers per nerve, fiber density, myelin area per nerve, myelinated fiber diameter, axon diameter, myelin thickness, or G-ratio.

The nerve impulse conduction speed was calculated using G ratio, a value representing the division of the axon's diameter by the nerve fiber diameter. According to Torch et al. 1989, G ratio between 0.6 and 0.79 was considered normal in nerve conduction. G ratio below 0.6 implies dense myelination, while the values above 0.79 evinces poor myelination and conduction abnormality. Quantification of Nissl-stained sensory neurons was performed according to the empirical method of Coggeshall et al., 1984. Briefly, nucleolar profiles (clearly visible nucleus with one or more nucleoli) were counted at 400X in every ten sections starting with a randomly chosen part. The calculations were multiplied by 10 and a correction factor to convert the nucleolar counts into neuronal numbers. This factor consisted of the nucleolar profile per 100 neurons after comparison of a randomly chosen section and the section adjacent to it. To study the dynamics of the lesion process, chromatolytic, eccentric, and pyknotic cells were quantified.

Statistical comparison

The Shapiro-Wilk was used for normality assessment. ANOVA was used for normally distributed data and SFI data. Friedman's test was used for intragroup comparison, and the Kruskal-Wallis test was used for intergroup comparison. The significance level was set at 5%.

RESULTS

Wharton's Jelly (WJ) is one of the sources of immuno-privileged stem cells and derived from extraembryonic mesoderm as neuro system. Hence, we attempted to study WJ as neural graft for regeneration of peripheral nerve injury. We approve that the human Wharton's jelly cells correspond phenotypically and functionally to MSCs. Accordingly, the cells were plastic adherent with fibroblastic morphology (Figure 1). Using flow cytometry analysis and immunocytochemistry, we found that mesenchymal stem cells isolated from Wharton jelly revealed the high cell surface expression of MSC markers CD105, CD90, CD73, CD44, and absence for hematopoietic cells (Figure 2).

The *in vivo* animal experiments were successfully carried out (Figure 3). The sciatic function index (SFI) values were standard by walking track analysis for both the test and control groups (Figure 4). The test and control animals were able to recover their sensation on the grafted legs by the end of two weeks. No mortality, signs of inflammations, and graft rejection were observed in Wistar rats during the surgical experiment and post-surgery (Figure 5). There was no evidence of fibrosis and neural scarring in both groups. Walking track analysis generated

a significant value, $P=0.523$, which implied similar healing and regeneration between the positive control and treated group (Figure 6). The immuno-histochemistry for T antigen CD25 (Figure 7) in explanted grafts indicates that WJ grafts are less immunogenic, although there was minimal infiltration of CD25 cells. The infiltration could be due to cross-species (human to rats) transplantation of graft. Moreover, no statistical difference in g-ratio was found between the autograft and WJ grafted group.

The functional nerve recovery was assessed by the rats walking track analysis. As the walking footprints are shown in Figure 4, the sciatic nerve injury treated group showed recovery. The SFI value varies from 100 to 0. The value 0 indicates normal function, and 100 means complete loss of function. The SFI value of treated group suggests functional recovery. Further, no significant difference exists between the autograft and test animal group. The interpersonal correlation in measurement was high after the 3rd week. Inadequate definition of footprints was noted in the first two weeks. The functional recovery results by pin-prick test, toe spread test, and sweating quantifications indicate that by the end of the 4th week, the test rats transplanted with umbilical cord have grade II recovery. In contrast, the autografted controls had better sensitivity (Table 1). MNG values were recorded for the first four weeks, and the values were noted (Table 2). The initial and final nerve action potentials were significant and showed gradual improvements from 1st week to 4th week. There was no significant difference in muscle weight between control and test animals. Trichome staining and microscopic analysis revealed that cross-section (C.S) of an intra-muscular nerve bundle and blood vessel formation are seen in treated sciatic nerve when compared to C.S of normal sciatic nerve. In the case of a longitudinal section of treated sciatic nerve, demarcation was observed between native sutured nerve and Wharton's jelly (Figure 6).

DISCUSSION

Clinical literature reports that less than 25% of nerve repair patients recover complete voluntary motor function and less than 3% recover full normal sensation [29]. The clinical results of these procedures have served as the impetus to continue the search for alternative methods of repairing peripheral nerve injuries. One such alternative is the use of various “nerve conduits” which may replace the need for nerve grafting. This is a novel attempt of using human umbilical cord Wharton's jelly (hUC WJ), which is discarded as medical waste as a biological nerve graft for peripheral nerve injury. This experiment is to validate the efficacy of using the human cord as a nerve graft, as it is the natural reservoir of immune privileged stem cells in Wharton jelly. The immunomodulatory effects of hUC-MSCs are mediated by multiple factors. The hUCMSCs

could reduce or block MHC class II expressions on vascular endothelial cell surface in cardiac allografts and decrease proinflammatory cytokines IL2 and IFN- γ , enhance anti-inflammatory cytokines, such as IL10 and TGF- β 1 expression *in vivo*. The soluble factors secreted by hUC-*MSC* have immunoregulatory effects as well [22]. For more than a decade there are several attempts exploring the use of umbilical cord as a graft for clinical application. In 1995, Angelo had conducted a large animal experiment using lamb autologous umbilical cord veins and arteries for femoral artery reconstructive surgeries. The preliminary results suggest that umbilical vessels could be used as vascular autograft provided with the immediate care of vasoactive graft during the perioperative period. Chen Y et al. 2012 hypothesize that the HUC may be an adequate novel substitute for the reconstruction of the extrahepatic bile duct.

WJ tissue is the combination of stromal microenvironment and hUC-*MSCs*, which could be appropriate for treating Traumatic brain injury (TBI) than hUC-*MSCs* cell suspension. Transplanting the human WJ tissue on the surface of the injured brain of Sprague Dawley rats developed significant protection with improved histologic and functional outcomes after TBI [5]. Decellularized human umbilical arteries seeded with human umbilical vein endothelial cells preserved the extracellular matrix and retained function *in vivo* for up to 8 weeks in nude rats. These properties suggest the potential use of decellularized umbilical arteries as small-diameter vascular grafts [12]. Recent research with decellularized Wharton's jelly (DWJ) as 3D tissue engineered graft that can be used as osteogenic or chondrogenic grafts. The decellularized Wharton jelly graft experiments in murine calvarial defect model with green fluorescent protein (GFP) labeled osteocytes indicates that this matrix can be used as a 3D porous, bioactive, and biocompatible scaffold for tissue engineering and regenerative medicine applications [17,18]. Moreover, Recent surgical advances make use of cryopreserved autologous umbilical cords as shunt conduits in neonatal cardiac surgery clinical trial (ClinicalTrials.gov Identifier: NCT02766998)

In our experiment, WJ sutured to the sciatic nerve transection showed functional recovery of rat model at the site of nerve injury. Axonal regeneration was observed during the explant after four weeks of surgery. Functional recovery was evidenced by walking track analysis and histopathological studies. Walking track analysis is a quantitative method of analyzing functional recovery of peripheral nerve in the rats. The performance of hind limbs after nerve injuries are analyzed by examining footprints, known as the SFI [32]. In our studies the performance and analysis infers the explanted tissue analysis shows similar healing between auto grafted and treated group. To add on histopathology evaluation had sections of developing axons and neoangiogenic vessels. Axonal regeneration may be aided due to the presence of *MSCs* in WJ.

The WJ-derived MSCs avoid ethical issues involved in embryonic stem cell research and being immune-privileged, which makes them an advantageous cell type for allogeneic transplantation [23,30]. The *in vitro* culture studies proved that these MSCs are plastic adherent cells positive for markers CD105, CD90, CD73, CD44 and could be differentiated to neuron-like cells. WJ-MSCs have the ability to differentiate into WJ-Schwann cells like cells (WJ-SCLCs) that effectively enhance the outgrowth of neurites *in vitro*. In addition, implantation of WJ-SCLCs-laden acellular nerve grafts (ANGs) isolated and decellularized from male Sprague–Dawley (SD) rats were superior to ANGs alone in inducing functional recovery after sciatic nerve injury [7]. A novel method using lyophilization and enzymes to prepare Acellular Nerve Scaffold (ANS) was effective in removal of cells, myelin and maintain the integrity and mechanical properties of nerve fiber than the traditional methods. The combination of hUC-MSCs with ANS promotes repair and regeneration of the sciatic nerve SD animal model [20,22]. In another similar studies Tonsils derived Mesenchymal stem cells (T-MSCs) were differentiated to Schwann cells *in vitro*. T-MSCs were able to express SC-specific markers, support of neurite outgrowth, and formation of myelin sheaths. Moreover, T-MSC-SC transplantation produced functional improvements in a mouse model of sciatic nerve mild injury [19].

WJ-MSCs may serve as a valuable source of SCs for use in transplantation, and human WJ-SCLCs may be effective for promoting the regeneration of peripheral nerves. Topical administration of hWJ-MSC bound to a delivery agent such as hydrogel may even provide sustained therapeutic effects with localized delivery of cells to the affected area [33]. For proper development of therapeutic approaches, it is essential to elucidate the possible mechanism by which stem cells play a pivotal role in aiding in functional recovery after peripheral nerve injury. We hypothesize that the therapeutic benefits such as angiogenesis, neurogenesis, synaptogenesis, cell fusion, and reduction of apoptosis could be achieved in the zone of the injury by implanting human umbilical cord. The elevation of neuronal-specific genes and varying levels of neural and astrocyte-specific proteins could be confirmed by immunolabelling after explantation of the graft. We envisage further elucidation of these ongoing investigations in the hope of using hUB WJ as nerve graft that will meet the complete clinical requirements.

Limitation of the study

It was challenging to suture the nanocoated umbilical cord with native rat sciatic nerve as it was very tiny. We should have used gelatin glue instead of suturing. However, the outcome of this experiment was excellent. All the experimental rats resumed the functional recovery, no immuno rejection was observed.

CONCLUSIONS

In conclusion, we emphasize that the in vivo animal experimentation of the human umbilical cord Wharton jelly devoid of vascular elements, allantoic duct, and amniotic epithelium can be a perfect neuro graft for peripheral nerve injuries. WJ is a natural reservoir of immune-privileged stem cells, which share properties of adult and embryonic stem cells should be a boon in nerve remodeling. Moreover, the presence of hyaluronic acid, collagen and growth factors guide the neurogenesis. The nerve repair and reconstruction data from animal experiments can be translated to clinical practice followed by clinical trials. However, some factors such as cognitive capacity and coping, which are known to influence outcome following nerve repair, are challenging to study in animal models. Furthermore, the molecular and cellular mechanism of neurogenesis needs to be analyzed using an immune deficient animal model. Future concurrent research is necessary to elucidate the use of cryopreserved umbilical cords as neural grafts as it has been a recent practice to bank umbilical cords along with cord blood.

Acknowledgements

The authors are grateful to Dr. Jaikanth Chandrasekaran, Ms. Lavanya and Ms. Vimala for the help in animal experiments. We also thank our PG Diploma students from Stem cell dept for their help in laboratory analysis.

Conflict of interest: None declared

REFERENCES

1. Angelo AV, Hovaguimian H, Arntson E, et al. Use of autologous umbilical artery and vein for vascular reconstruction in the newborn. *J thorac cardiovasc surg.* 1995; 109: 854-857. DOI: 10.1016/S0022-5223(95)70308-X . PMID: 7739244
2. Bain J R, Mackinnon S E, Hunter DA. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. *Plast. Reconstr. Surg.* 1989; 83 : 129–138. doi: 10.1097/00006534-198901000-00024. PMID: 2909054
3. Barker RA, Barrett J, Mason SL, et al. Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. *Lancet Neurol.* 2013; 12:84-91. doi: 10.1016/S1474-4422(12)70295-8.

4. Campagnoli C, Roberts IA, Kumar S, et al. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood*. 2001;98:2396–2402. doi: 10.1182/blood.v98.8.2396. PMID: 11588036
5. Cheng T, Yang B, Li D, et al. Wharton's Jelly Transplantation Improves Neurologic Function in a Rat Model of Traumatic Brain Injury. *Cell Mol Neurobiol*. 2015; 35: 641–649. doi:10.1007/s10571-015-0159-9. PMC 4481175 . PMID 25638565.
6. Cheng Y, Lin Y , Xiong X, et al. The human umbilical cord: A novel substitute for reconstruction of the extrahepatic bile duct. *Journal of Medical Hypotheses and Ideas*. 2012; 6: 7–11. doi:http://dx.doi.org/10.1016/j.jmhi.2012.03.001
7. Choi SJ, Park SY, Shin YH, et al. Mesenchymal Stem Cells Derived from Wharton's Jelly Can Differentiate into Schwann Cell-Like Cells and Promote Peripheral Nerve Regeneration in Acellular Nerve Grafts. *Tissue Eng Regen Med*. 2021;18(3):467-478. doi: 10.1007/s13770-020-00329-6. PMID: 33515168;
8. Coggeshall R E, Chung K, Greenwood D, et al. An empirical method for converting nucleolar counts to neuronal numbers. *J Neurosci Methods*. 1984; 12:125–132. DOI: 10.1016/0165-0270(84)90011-6 . PMID: 6527551
9. Cudlip SA, Howe F A, Griffiths J R, et al. Magnetic resonance neurography of peripheral nerve following experimental crush injury, and correlation with functional deficit. *Journal of neurosurgery*.2002; 96: 755-759. .DOI: 10.3171/jns.2002.96.4.0755. PMID: 11990818
10. De Medinaceli L, Freed WJ, Wyatt R J. An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. *Exp. Neurol*. 1982; 77:634–643. DOI: 10.1016/0014-4886(82)90234-5 . PMID: 7117467
11. Dvir T, Timko BP, Kohane DS, et al. Nanotechnological strategies for engineering complex tissues. *Nat Nano*.2011; 6: 13-22. DOI: 10.1038/nnano.2010.246. PMCID: PMC4059057
12. Gui L, Muto A, Chan SA, Breuer CK, et al. Development of Decellularized Human Umbilical Arteries as Small-Diameter Vascular Grafts. *Tissue Eng Part A*.2009;15: 2665–2676. doi: 10.1089/ten.tea.2008.0526. PMID: 19207043
13. Houle JD, and Reier PJ. Transplantation of Fetal Spinal Cord Tissue Into the Chronically Injured Adult Rat Spinal Cord. *Journal of Comparative Neurology*.1988; 269:535-547. doi: 10.1002/cne.902690406. PMID: 2453536.
14. Hsieh JY, Wang HW, Chang SJ, et al. Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and

- angiogenesis. PLOS one.2013; 8 .e72604. doi: 10.1371/journal.pone.0072604. PMID: 23991127
15. Hunt SP, Mantyh PW. The molecular dynamics of pain control. *Nat Rev Neurosci.* 2001;2 :83–91. doi: 10.1038/35053509. PMID: 11252998.
 16. Iftimia-Mander A, Hourd P, Dainty R, et al. Mesenchymal stem cell isolation from human umbilical cord tissue: understanding and minimizing variability in cell yield for process optimization. *Biopreservation Biobank.* 2013;11: 291-298. doi: 10.1089/bio.2013.0027. PMID: 24835260.
 17. Irina A, Timur F, Evgeniya K, et al. Role of VEGF-A in angiogenesis promoted by umbilical cord-derived mesenchymal stromal/stem cells: in vitro study, *Stem Cell Research & Therapy* (2016) 7:46 DOI 10.1186/s13287-016-0305-4
 18. Jadalannagari S, Cinverse G, McFall C, et al. Decellularized wharton’s Jelly from human umbilical cord as a novel 3D scaffolding material for tissue engineering applications. *PLoS ONE* 2017;29, e0172098. doi: 10.1371/journal.pone.0172098. PMID: 28222169
 19. Jung N, Park S, Choi Y, Park JW, et al. Tonsil-Derived Mesenchymal Stem Cells Differentiate into a Schwann Cell Phenotype and Promote Peripheral Nerve Regeneration. *Int J Mol Sci.* 2016;17(11):1867. doi: 10.3390/ijms17111867. PMID: 27834852.
 20. Marinescu SA, Zărnescu O, Mihai IR, et al. An animal model of peripheral nerve regeneration after the application of a collagen-polyvinyl alcohol scaffold and mesenchymal stem cells. *Rom J Morphol Embryol.* 2014;55(3):891-903. PMID: 25329117.
 21. Place E S, Evans N D, Stevens M M. Complexity in biomaterials for tissue engineering. *Nat Mater.* 2009;8:457-470. doi: 10.1038/nmat2441. PMID: 19458646.
 22. Qian C, Zhang Z, Zhao R, et al. Effect of acellular nerve scaffold containing human umbilical cord-derived mesenchymal stem cells on nerve repair and regeneration in rats with sciatic nerve defect. *Ann Transl Med.* 2022;10(8):483. doi: 10.21037/atm-22-1578. PMID: 35571424.
 23. Qiu Y, Yun MM, Han X, et al. Human umbilical cord mesenchymal stromal cells suppress MHC class II expression on rat vascular endothelium and prolong survival time of cardiac allograft. *Int J Clin Exp Med.* 2014;7(7):1760-7. PMID: 25126177.
 24. Ramesh B, Bishi DK, Rallapalli S, et al, Ischemic cardiac tissue conditioned media induced differentiation of human mesenchymal stem cells into early-stage

- cardiomyocytes. *Cytotechnology* 2012; 64 (5), 563-575. doi: 10.1007/s10616-012-9440-7. PMID: 22395895
25. Ramesh B, Chandrasekaran J, Jeevankumar S, et al. (2017) Hybrid Amniotic Membrane Dressing with Green Silver Nanoparticles as Bioengineered Skin for Wounds and Burns: A Pilot Studies. *J Biotechnol Biomater* 7: 272. doi: 10.4172/2155-952X.1000272
26. Ramesh B, Kotturathu MC, Adegbenro O J F, Fabrication and Electrospinning of 3D Biodegradable Poly-l-Lactic Acid (PLLA) Nanofibers for Clinical Application, *Stem Cell Nanotechnology Methods and protocols*, 2020 ;2125:119-128. doi: 10.1007/7651_2019_213. PMID: 30771191.
27. Schiaveto de Souza A, da Silva CA, Del Bel EA. Methodological Evaluation to Analyze Functional Recovery after Sciatic Nerve Injury. *Journal of Neurotrauma*. 2004; 21: 627-635. doi: 10.1089/089771504774129955. PMID: 15165370.
28. Siddique R, Thakor N. Investigation of nerve injury through microfluidic devices. *J. R. Soc. Interface*.2014; 11: 20130676. doi: 10.1098/rsif.2013.0676. PMID: 24227311
29. Smit, Xander. (2006). Struggle at the site of nerve injury: a rat sciatic nerve study on fundamental problems of peripheral nerve injury. ISBN-10: 90-9020531-4
https://repub.eur.nl/pub/8033/061013_Smit,%20Xander.pdf
30. Taghizadeh RR, Cetrulo KJ, Cetrulo CL. Wharton's Jelly stem cells: future clinical applications. *Placenta*. 2011;4:S311-5. doi: 10.1016/j.placenta.2011.06.010. PMID: 21733573
31. Torch S, Usson Y, Saxod R. Automated Morphometric Study of Human Peripheral Nerves by Image Analysis. *Pathology - Research and Practice*.1989;185:567-71. doi: 10.1016/S0344-0338(89)80195-5. PMID: 2626366
32. Varejão AS, Meek MF, Ferreira AJ, et al. Functional evaluation of peripheral nerve regeneration in the rat: walking track analysis. *J Neurosci Methods*. 2001;108(1):1-9. doi: 10.1016/s0165-0270(01)00378-8. PMID: 11459612.
33. Wang AYL, Loh CYY, Shen HH, et al. Topical Application of Human Wharton's Jelly Mesenchymal Stem Cells Accelerates Mouse Sciatic Nerve Recovery and is Associated with Upregulated Neurotrophic Factor Expression. *Cell Transplant*. 2019 Dec;28(12):1560-1572. doi: 10.1177/0963689719880543. PMID: 31565957
34. Zhao Z, Wang Y, Peng J, et al. Repair of nerve defect with acellular nerve graft supplemented by bone marrow stromal cells in mice. *Microsurgery*.2011; 31:388-394. doi: 10.1002/micr.20882. PMID: 21503972

Table 1: Functional Evaluation for Control and Test animals

S.No.	Functional evaluation	1st week		2nd week		3rd week		4th week	
		Control rats	Test rats	Control rats	Test rats	Control rats	Test rats	Control rats	Test rats
1	Pin-prick test	Grade I	Grade 0	Grade I	Grade 0	Grade II	Grade I	Grade III	Grade II
1	Toe spread test	Grade 0	Grade 0	Grade I	Grade 0	Grade I	Grade I	Grade II	Grade II
3	Sweating quantification [μ L]	9.75 \pm 1.7	7 \pm 1.4	11 \pm 1.8	8.25 \pm 1.2	14.25 \pm 0.9	11 \pm 1.8	16 \pm 1.4	12.25 \pm 2.0

Table 2. Action potential of sciatic nerve evaluation using MNG

MNG	1st week		2nd week		3rd week		4th week	
	Control rats	Test rats	Control rats	Test rats	Control rats	Test rats	Control rats	Test rats
LATN1 [ms]	0.12 \pm 0.09	0.8 \pm 0.18	0.17 \pm 0.16	0.11 \pm 0.12	0.22 \pm 0.08	0.15 \pm 0.14	0.28 \pm 0.15	0.23 \pm 0.12
LATN2 [ms]	0.18 \pm 0.06	0.9 \pm 0.10	0.26 \pm 0.12	0.18 \pm 0.15	0.30 \pm 0.16	0.23 \pm 0.14	0.36 \pm 0.10	0.43 \pm 0.12
CVN1 [m/s]	62 \pm 26.75	55 \pm 28.05	65 \pm 32.1	58 \pm 25.3	70.25 \pm 28.13	63 \pm 18.2	85 \pm 23.3	69 \pm 35.8
CVN2 [m/s]	46 \pm 24.3	33 \pm 16.8	54.31 \pm 23.5	39.64 \pm 28.5	66.32 \pm 22.06	43.28 \pm 26.71	79.24 \pm 17.8	52.21 \pm 22.7
AMPN1 [mV]	1.12 \pm 0.32	0.83 \pm 0.46	1.38 \pm 0.26	0.9 \pm 0.54	1.57 \pm 0.48	1.39 \pm 0.36	1.8 \pm 0.43	1.43 \pm 0.34
AMPN2 [mV]	0.52 \pm 0.28	0.31 \pm 0.35	0.68 \pm 0.33	0.43 \pm 0.26	0.74 \pm 0.49	0.61 \pm 0.47	0.86 \pm 0.34	0.77 \pm 0.29

AMPN1 — initial nerve action potentials amplitudes; AMPN2 — final nerve action potential amplitudes; LATN1 — initial nerve action potentials latencies; LATN2 — final nerve action potential latencies; CVN1 — initial nerve action potential conduction velocities; CVN2 — final nerve action potentials conduction velocities; ms — millisecond; m/s — meters per second; mV — millivolt

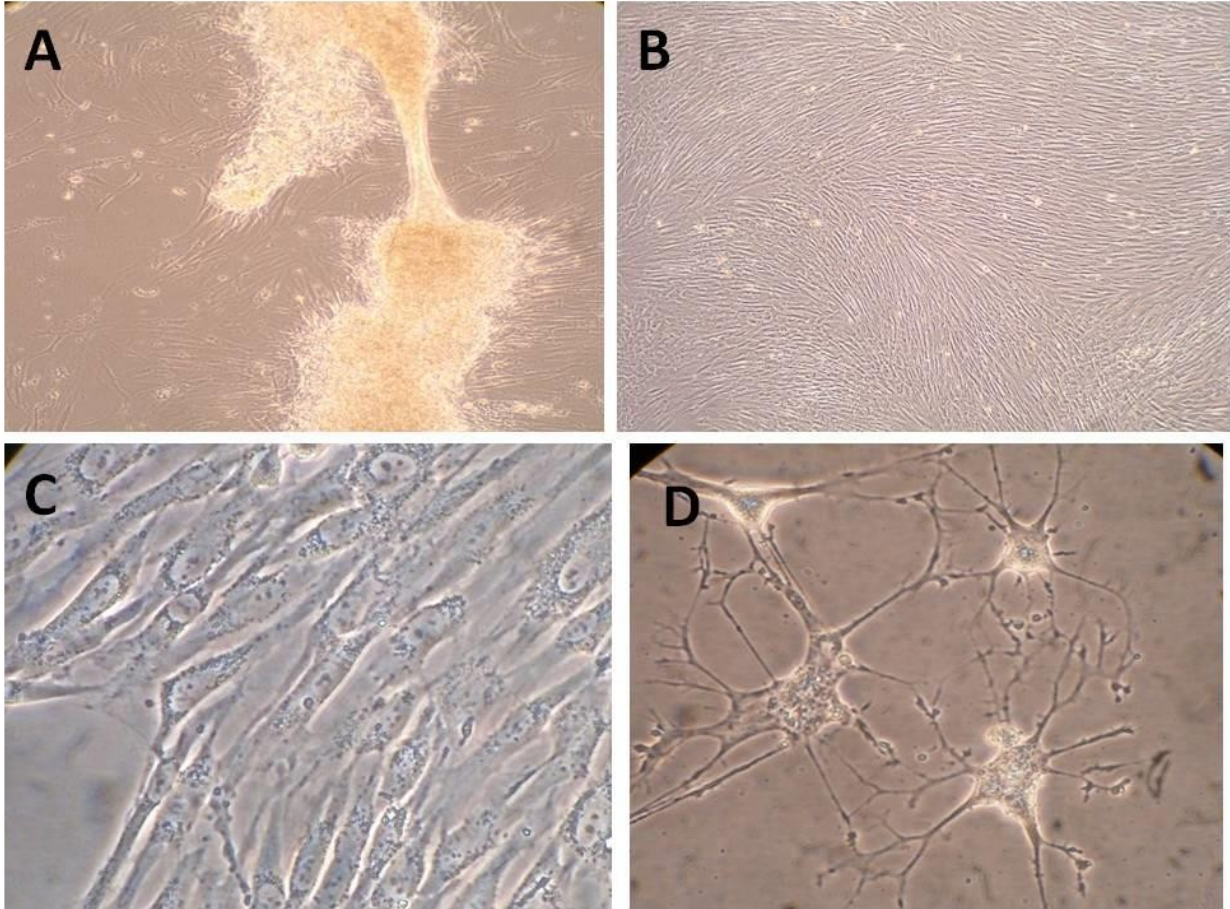


Figure 1. Expansion and differentiation of Umbilical cord derived stem cells, **A.** Primary culture of collagenase digested umbilical cord stem cells; **B.** Passaged mesenchymal stem cells (x40); **C:** P4 UCMSCs control undifferentiated cells (x400); **D:** UCMSCs differentiated to neuron like cells (x400).

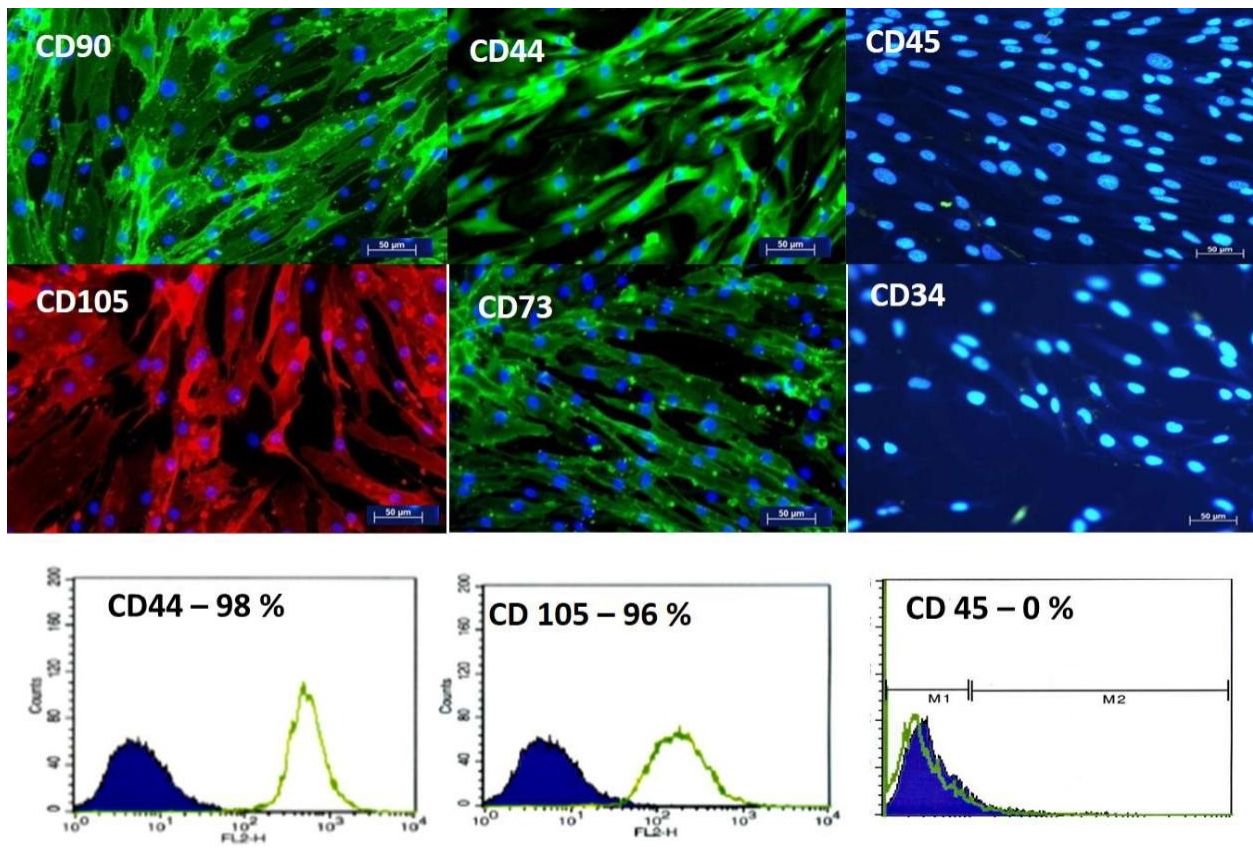


Figure 2. Immunohistochemistry and flowcytometry; immunocytochemistry and flowcytomerty analysis of stem cells isolated from Wharton’s jelly. Red color — Tetramethyl Rhodamine, Green color — Fluorescein isothiocyanate (FITC), Blue color — 4’6-diamidino phenylindole (DAPI) stain for nucleus.

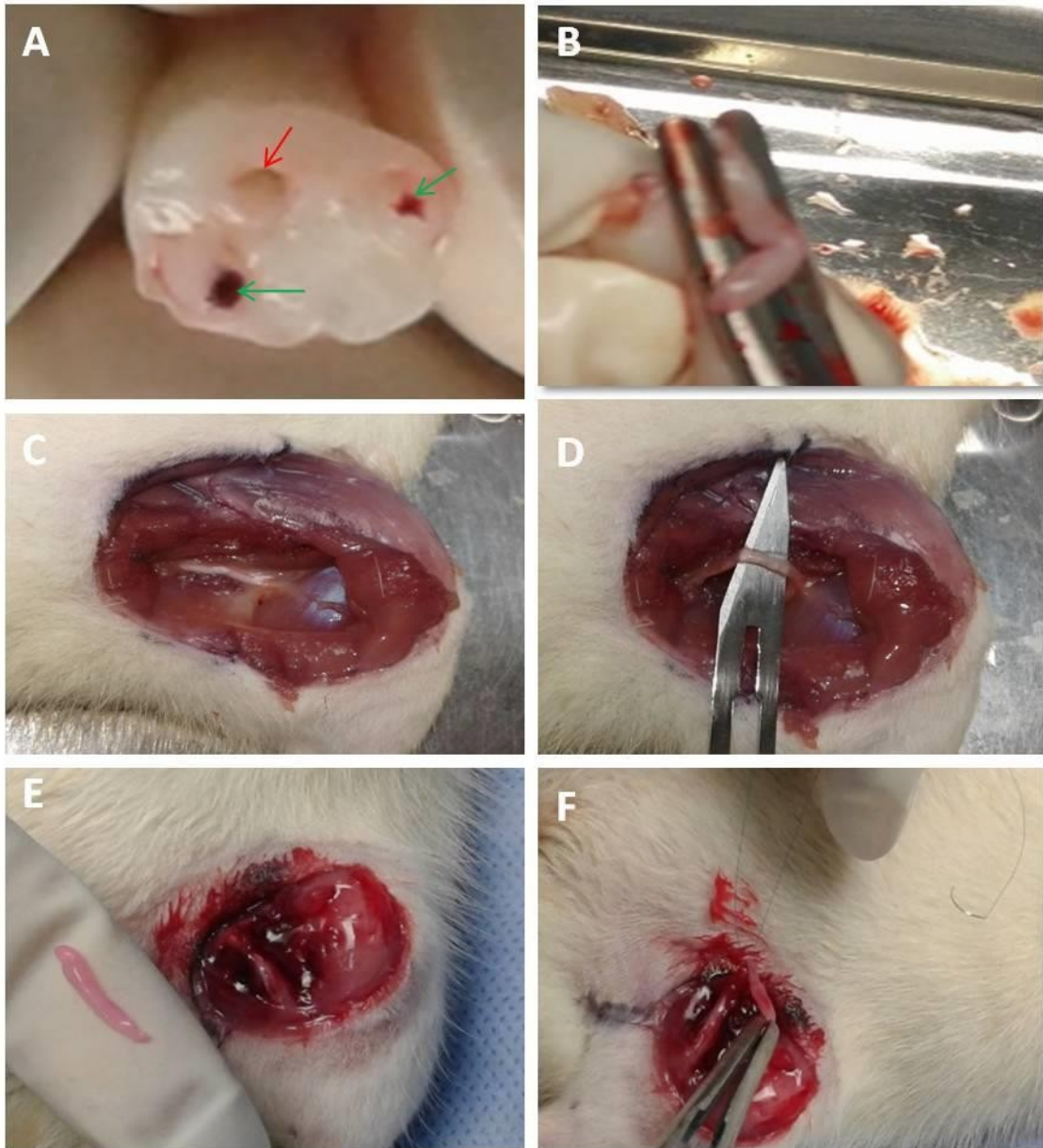
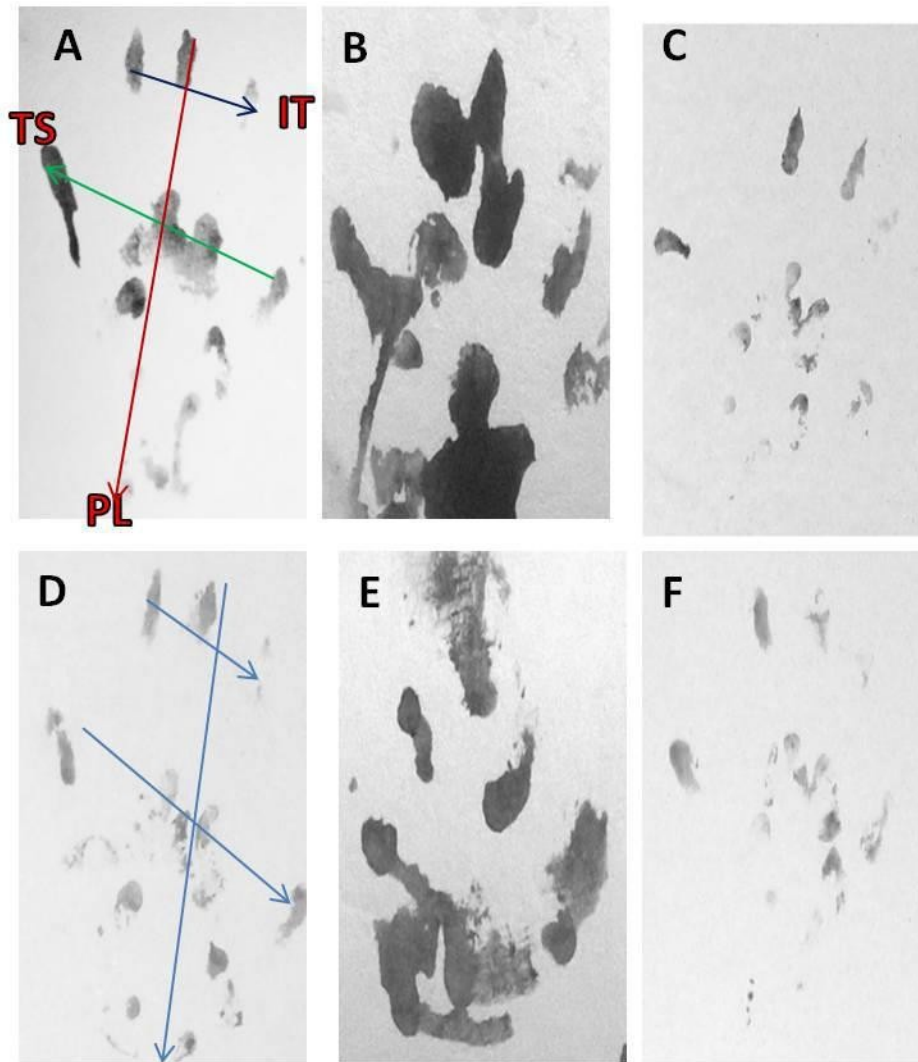


Figure 3. Sciatic nerve animal experiments, in vivo animal experiments; **A.** Cross section view of human umbilical cord showing one umbilical vein (red color arrow) and two arteries (green arrows); **B.** Dissection of umbilical portion devoid of vascular elements; **C.** Exposure of left sciatic nerve; **D.** Separation of left sciatic nerve; **E.** Trimming the umbilical cord to the size of nerve; **F.** Suturing the umbilical cord portion to the cut end of the nerve.



$$SFI = -38.3 \times \left(\frac{EPL - NPL}{NPL} \right) + 109.5 \times \left(\frac{ETS - NTS}{NTS} \right) + 13.3 \times \left(\frac{EIT - NIT}{NIT} \right) - 8.8$$

Figure 4. Walking track analysis; Walking track analysis image, **A, B, C.** Control mice before autografting, 2 week postgrafting, 4 week post grafting; **D, E, F.** Test rat before Wharton jelly grafting, 2 week post grafting, 4 weeks post grafting; IT — intermediate toe spread; TS — Toe spread; PL — print length.



Figure 5. Explanted nerves; Formaldehyde fixed explanted nerves after 2 months of implantation, **A.** Control Sciatic nerve; **B.** Autografted control nerve; **C.** Umbilical cord grafted portion.

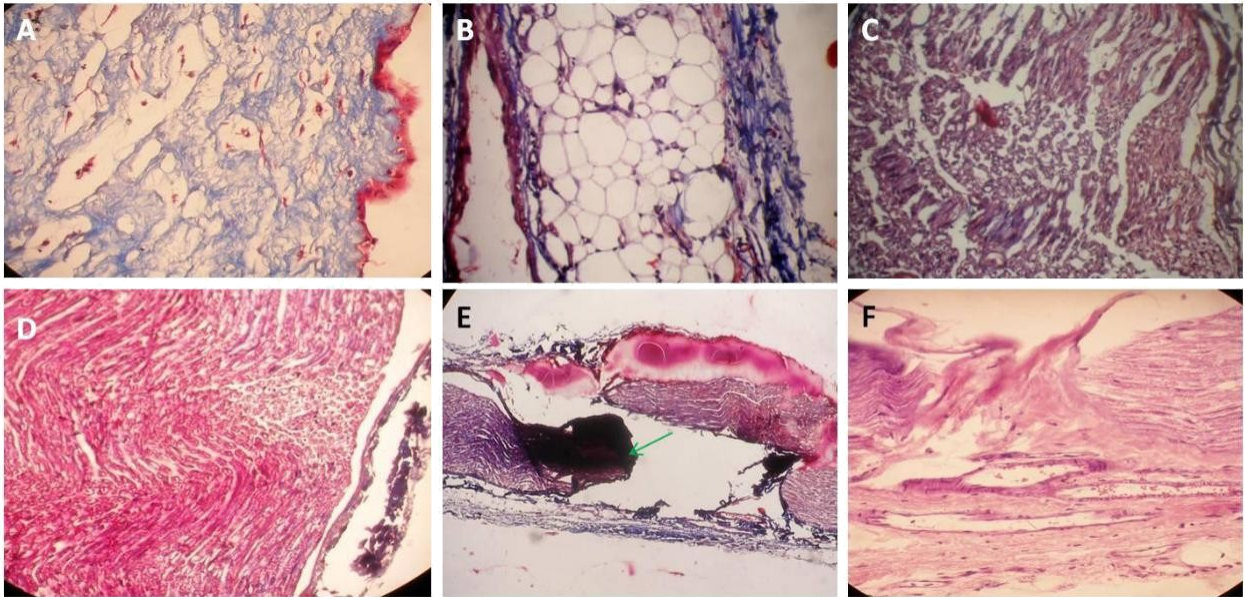


Figure 6. Histopathological Trichrome staining for explanted nerves; **A.** Control umbilical cord prior to implantation; **B.** One month explanted rejected tissue; **C.** Two months old explanted umbilical cord tissue; **D.** Control sciatic nerve; **E.** Autografted Sciatic nerve showing the axonal growth cone at site of axotomy; **F.** Two months old autografted nerve at the site of axotomy.

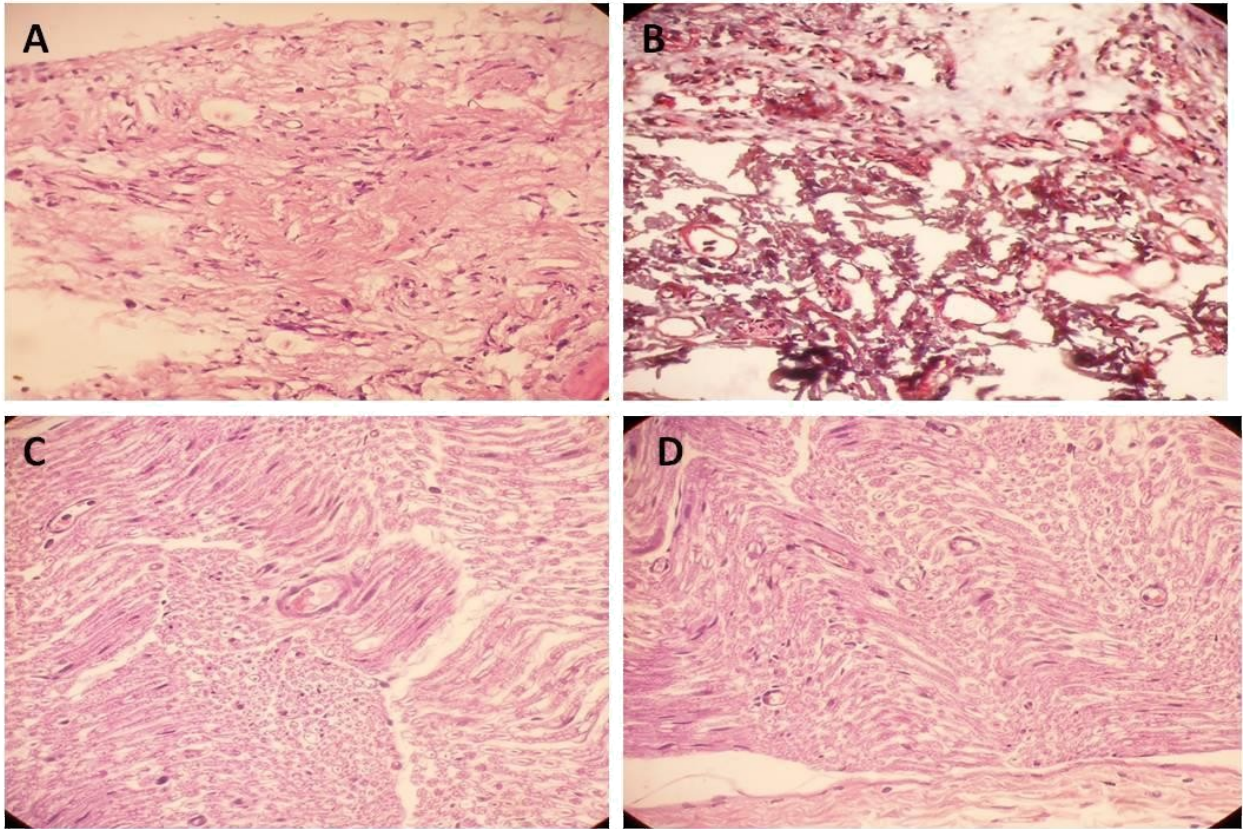


Figure 7. Immunohistochemistry for infiltration of CD25 activated T cells; **A.** Control umbilical cord-negative for CD25; **B.** Explanted umbilical cord positive for CD25; **C.** Control native sciatic nerve- negative for CD25; **D.** Immunostained sciatic nerve explanted control-negative for CD25.