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Effect of Citicoline Loaded PHBA Tubular Grafts on Axonal Regeneration in Sciatic Nerve Injury : An Experimental Approach as an Autograft Alternative

Hıdır Özer,[1] Ali Yılmaz,[1] Mürüvvet Akçay Çelik,[2] Yeliz Kaşko Arıcı,[3] Vugar Nabi,[4] Ebru Erdal,[5] Songül Özay,[6] Murat Demirbilek,[6] Mehdi Hekimoğlu[7]

Department of Neurosurgery,[1] Faculty of Medicine, Ordu University, Ordu, Turkey
Department of Medical Pathology,[2] Faculty of Medicine, Ordu University, Ordu, Turkey
Department of Biostatistics and Medical Informatics,[3] Faculty of Medicine, Ordu University, Ordu, Turkey
Department of Orthopaedics and Traumatology,[4] Antalya Training and Research Hospital, University of Health Sciences, Ministry of Health, Antalya, Turkey
Advanced Technologies Application and Research Center,[5] Faculty of Medicine, Ankara Yıldırım Beyazıt University, Ankara, Turkey
Department of Biology,[6] Faculty of Arts and Sciences, Ankara Hacı Bayram Veli University, Ankara, Turkey
Department of Neurosurgery,[7] American Hospital, Istanbul, Turkey

Objective : This study aims to evaluate the effects of poly(3-hydroxybutyrate)/alginate (PHBA/ALG) tubular graft containing cytidine 5′-diphosphocholine (citicoline) on axonal regeneration in an experimental rat sciatic nerve injury model and to compare the results of this method with autologous nerve grafting.

Methods : A total of 24 Wistar-Hannover albino rats were randomly divided into three groups (n=8). In all animals, a 10 mm segment was removed from the right sciatic nerve under microscope. The nerve defect was repaired using autologous nerve graft (group O), PHBA graft only (group T), and PHBA graft containing citicoline (group S). Sixteen weeks later, all animals were euthanized and sciatic nerve samples were taken for histopathological examination. Sections were stained with hematoxylin-eosin and Masson Trichrome.

Results : The groups were compared statistically in terms of regenerated axon diameter, number of blood vessels, nerve sheath degeneration, edema, inflammatory cell infiltration, and necrosis. Histopathological evaluation showed that regenerative axons were formed in all three groups. PHBA and citicoline-containing PHBA grafts successfully bridged the damaged area. While the best results were obtained in the autograft group, the results of the PHBA group containing citicoline were statistically significantly better than the PHBA group. In general, the distribution of histopathological parameters showed that citicoline administration showed protective effects on parameters such as inflammation and cell death. Axon diameter : group S had significantly larger axon diameters than group T ($p<0.05$). Number of blood vessels : the highest values were in the autograft group and the difference between groups O and T was significant ($p<0.05$).

Conclusion : PHBA-based tubular graft with oriented nanofiber structure containing citicoline supports peripheral nerve regeneration. Although our results are not better than autograft, it may be a promising alternative for cases where autologous graft is not available.

Key Words : Peripheral nerves · Nerve regeneration · Polyhydroxybutyrate · Alginates · Cytidine diphosphate choline.

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• Address for correspondence : **Mehdi Hekimoğlu**
Department of Neurosurgery, American Hospital, Teşvikiye Mahallesi, Güzelbahçe Sokak No. 20, Şişli, İstanbul 34365, Turkey
Tel : +90 4443777, Fax : +90 4443777, E-mail : mehdih@amerikanhastanesi.org, ORCID : https://orcid.org/0000-0002-1182-2216

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INTRODUCTION

Peripheral nerve traumas that lead to motor and sensory loss in patients remain a significant public health concern[2]. Repair with autologous nerve grafts is the undisputed gold standard for treating nerve gap injuries. However, since autograft repair is not always feasible, alternative reconstruction strategies have been developed. In recent years, nerve conduits made from synthetic biomaterials have been introduced to address this need and have been widely employed in preclinical models[5]. Nevertheless, the use of these synthetic grafts presents several limitations, including peripheral scar tissue formation, neuroma development, and graft-tissue diameter mismatch[44]. In order to overcome these difficulties, biocompatible and biodegradable polymers have been focused on and especially the effects of poly-3-hydroxybutyrate (PHB) based channels on peripheral nerve regeneration have been observed to give successful results in rat and rabbit models[38,43]. One experimental study also demonstrated that PHB is a reliable material that can be used as an alternative to conventional toxic plastics[37]. PHB is a biodegradable and non-toxic polymer synthesized by bacteria, functioning as an intracellular carbon and energy reserve[3]. For a synthetic tubular graft to successfully repair nerve injury, it must possess adequate mechanical strength and an appropriate lumen[34]. Although synthetic polymers used in tissue engineering offer improved mechanical properties and structural stability, natural polymers have gained attention due to their high biocompatibility and inherent biodegradability[6].

Biomaterials such as alginate (ALG), gelatin, and collagen are noteworthy for their cell compatibility and regenerative signaling properties. The addition of fibronectin to ALG hydrogel has been shown to enhance peripheral nerve regeneration by improving Schwann cell viability and axonal elongation[29]. Furthermore, the development of electrically conductive and flexible nanofibrous scaffolds incorporating the ALG molecule has been reported to simultaneously deliver mechanical and electrical cues to support nerve cell function[15].

In addition to these biomaterial-based approaches, the local and controlled release of pharmacological agents to promote nerve healing has attracted considerable interest in recent years. One such agent, cytidine 5′-diphosphocholine (citicoline), is notable for its neuroprotective, anti-inflammatory, and membrane-stabilizing effects. It has also been shown that the mortality rate of patients with cerebrovascular damage receiving citicoline treatment decreased[46]. The role of citicoline in choline metabolism has been extensively studied, particularly in ischemic cerebrovascular diseases, due to its multifaceted effects such as maintaining neuronal function, enhancing neuroregeneration, and modulating immune responses[45].

In this study, the regenerative effects of citicoline-loaded PHBA/ALG tubular grafts were investigated in an experimental peripheral nerve injury model. The outcomes were compared with autografts in terms of both histopathological and functional aspects. To the best of our knowledge, no previous *in vivo* study has examined the use of citicoline-loaded PHBA grafts.

MATERIALS AND METHODS

Approval for the study was granted by Ordu University Animal Research Ethics Commitee. All experimental procedures were carried out in the research laboratories. The study protocol was approved by the Local Ethical Committee of Experimental Animals on December 9, 2024, with the IACUC approval No. 82678388, date : December 9, 2024. The animals used in the study were obtained from Experimental Animals and Research Centre after obtaining official ethical committee permission.

PHB, ALG, chloroform, acetic acid, fluoroacetic acid, dimethyl sulfoxide (DMSO), and MTT (formazan salt), NaOH, epichlorohydrin (EPC), HCl, and sodium carboxymethylcellulose (CMC; medium viscosity) were purchased from Merck (Kenilworth, NJ, USA). Citicoline was supplied by Scientific Laboratory Supplies (Notingham, UK). The cell culture medium (DMEM/F-12), fetal bovine serum, and cell culture flasks were obtained from Gibco (Grand Island, NY, USA). L929 cells were acquired from the Sap Institute of the Ministry of Agriculture and Forestry (Ankara, Turkey).

Animal procedure

A total of 24 adult male Wistar-Hannover albino rats, aged 3 months and weighing between 225–275 g, were used in the study. The animals were housed individually in standard plastic cages under controlled conditions : ambient temperature of 22–25°C, stable humidity, and a natural light/dark cycle. To reduce stress that might affect experimental outcomes, the animals were allowed a 2-week acclimatization period, during which

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they were regularly handled. All animals had free access to standard laboratory rodent chow and tap water.

Conduit fabrication

Production of PHB and PHBA tubular grafts

To prepare the fiber membranes, 5% (weight/volume, w/v) PHB was dissolved in a chloroform and fluoroacetic acid solution (10 : 1 volume/volume [v/v]) at 60°C. The solution was then placed into a 20 mL injector. The injector pump speed was set to 5 μL/h. A covered copper collector was used, and the distance between the injector and the collector was maintained at 10 cm. The power supply was adjusted to 10 kV. After the electrospinning process, the membrane was kept in a hood overnight[1]. PHB membranes were coated with ALG using a 1% alginate solution prepared in pure water. The fibrillar PHB membranes were immersed in the alginate solution, allowing them to interact thoroughly. After this interaction, the membranes were dried under a fume hood. Subsequently, the dried membranes were cross-linked using a mixture of 0.32% gluconolactone (w/v) and 0.64% sodium carbonate (NaCO3, w/v) in a 2 : 1 methanol to water (v/v) solution[22]. ALG coated and uncoated membranes were sterilized with ethylene oxide and stored at 2–4°C until the day of use. After the PHBA structure membranes were produced, they were macroscopically shaped into tubular grafts. In this study, ALG coated PHB tubular grafts were named PHBA. Macroscopic images of the tubular grafts are presented in Fig. 1A and B.

Scanning electron microscopy (SEM) examinations

The physical structure of the PHB and PHBA membranes, as well as the microscopic characteristics of the ALG coating, were examined using SEM. Prior to SEM analysis, the membranes were coated with a 7 nm thick layer of gold-palladium.

Water retention and degradation studies of membranes

The water retention and *in vitro* degradation properties of PHB and PHBA membranes were assessed using the gravimetric method. For this study, the membranes were cut into 1×1 cm squares and weighed. Next, 5 mL of phosphate-buffered saline (PBS) at pH 7.4 was added to each membrane, which was then incubated for 1 hour at 37°C. After incubation, the membranes were washed and placed back in PBS for further incubation. The membranes were weighed once a week, and their weights were recorded during this period[20]. The degradation study was conducted over a period of 6 weeks. The water retention capacities of the membranes were determined using the following equation : swelling capacity (%) = A0 / A1 × 100. In the previous equation, A0 represents the dry weight of the membranes, while A1 denotes the weight after incubating the membranes in PBS for 1 hour. The *in vitro* degradation of the membranes was assessed using the following equation : degradation (%) = A1 / A2 × 100, where A1 is the wet weight of the membranes and A2 is the weight measured at specified intervals.

Mechanical properties of membranes

The tensile strength properties of PHB and PHBA membranes were assessed using a universal mechanical testing device. For this analysis, membranes measuring 1×5 cm of both PHB and PHBA were utilized. In the study, the testing speed was set at 10 mm/min, the preload (F0) was specified as 0.01 N, and the preload speed was established at 2 mm/min.

Protein adhesion of membranes

The protein adhesion properties of PHB and PHBA membranes were assessed using Coomassie blue reagent. For this purpose, square-shaped membranes measuring 1×1 cm were incubated in a 250 μg/mL solution of human serum albumin for 2 hours at 37°C. Following the incubation, the membranes were gently washed with distilled water and then incubated in 250 μL of a 0.5% Triton X-100 solution for 30 minutes at 37°C. Finally, the amount of protein in the buffer solution was measured[30]. A calibration curve was created using human serum albumin solutions at varying concentrations.

Cell culture studies

In this study, cells derived from mouse abdominal connective tissue were utilized. The cells were cultured in DMEM-F12 medium supplemented with 10% fetal calf serum at 37°C in an incubator with 5% carbon dioxide. To determine the ratio of cells cultured on the membranes, the membranes were placed in 24-well plates. A total of 1×10^3 cells per mL was seeded on the membranes and incubated at 37°C. After 1, 3, 5, and 7 days of incubation, the concentration of cells adhering to the membranes was assessed using the MTT assay, measured in terms of absorbance. On the specified days, the medium was discarded, and an MTT solution (5 mg/mL) diluted 1 : 10 (v/v) with medi-

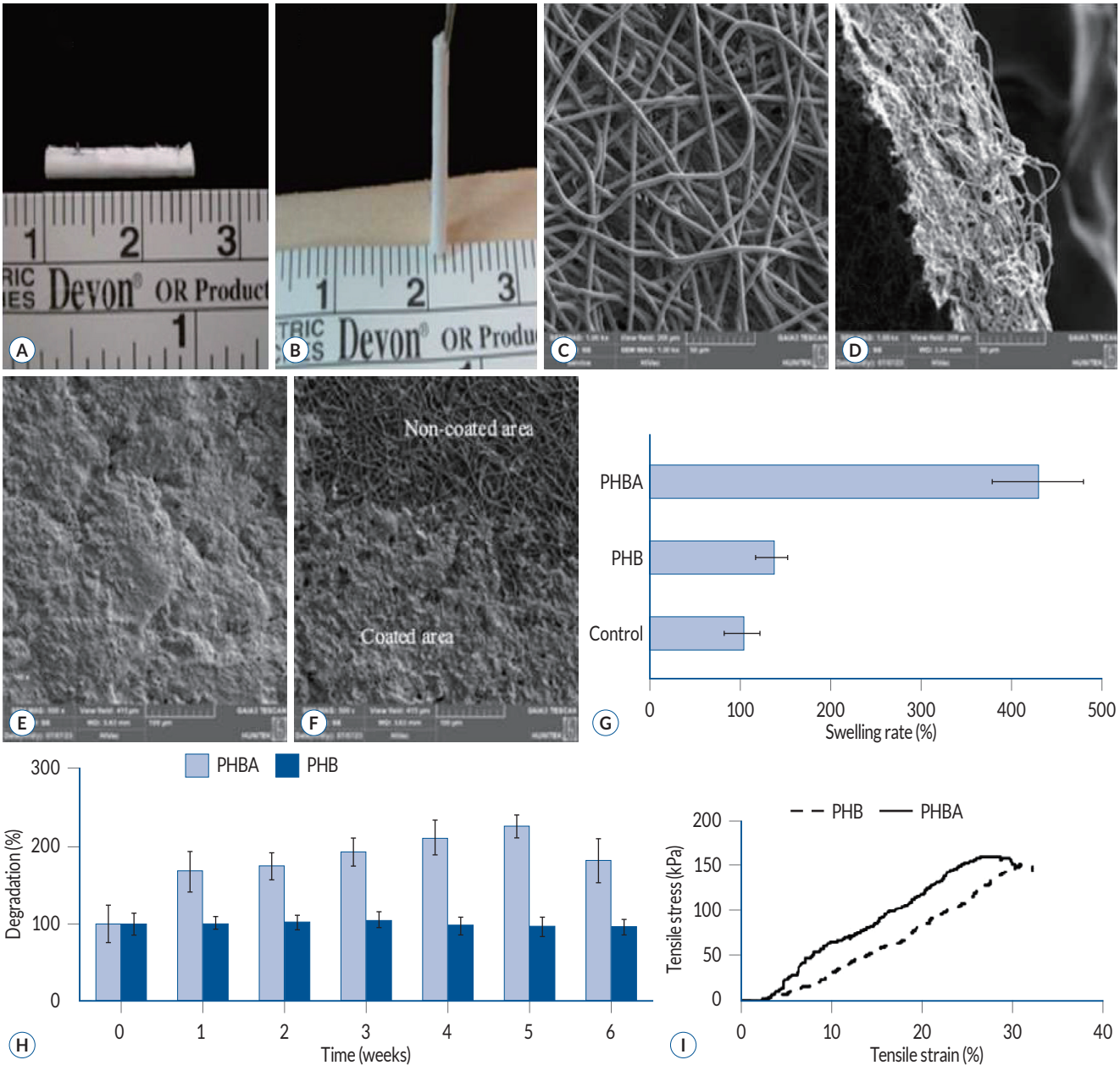


Fig. 1. Characterization of PHB and PHBA tubular grafts. Horizontal (A) and vertical (B) view of PHBA tubular grafts. Horizontal (C) and vertical (D) scanning electron microscopy (SEM) view of PHB fiber membrane. Horizontal SEM view of alginate (ALG) coated (E) and half coated PHBA membrane (F). Swelling (G) and degradation profile (H) of the PHB and PHBA membranes were determined by gravimetric method. n=6, $p<0.01$. I : Mechanical properties of the PHB and PHBA membrane were determined via stress-strain curve. PHB : poly(3-hydroxybutyrate), PHBA : poly(3-hydroxybutyrate)/alginate.

um was added to each well. After 4 hours of incubation, the solution was discarded, and 200 μL of DMSO was added to each well. The absorbance of the dissolved formazan crystals was then measured at 570 nm. Membrane extracts were obtained following ISO standards[27]. To obtain the extract, a 6 cm^2 membrane was incubated in 1 mL of medium for 72 hours at 37°C. The oxidative damage to the DNA of membranes was assessed by measuring the amount of 8-hydroxy-2'-deoxyguanosine (8OHdG). For this purpose, L929 cells were seeded in 24-well plates at a concentration of 10×10^3 cells/mL and a volume of 1 mL. The cells were then incubated with the sample extracts for 48 hours. After incubation, the cells in the plates were gently

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washed with distilled water. Next, 250 μL of distilled water was pipetted into each well, and the cells were lysed using the freeze-thaw method. The liquid phase from the plates was collected and centrifuged at 5000 rpm for 10 minutes. The amount of 8OHdG in the samples was then determined using a commercial kit. The cells treated with standard medium were used as negative controls in this study.

Preparation of citicoline gel

To prepare the citicoline-loaded CMC gels, 50 mg of citicoline was dissolved in 5 mL of 5% (v/w) CMC solution, along with 1% (w/v) urea and 1% (w/v) NaOH. Next, 1% (v/v) EPC in 1 mL of DMSO was added to the mixture. The solution was stirred in the dark for 24 hours at 100 rpm at room temperature to promote gelation. After this, the pH of the gel was neutralized using 1% (v/v) HCl. The gel was washed twice with PBS to eliminate any unbound EPC, CMC, and citicoline[39]. The swelling and degradation profiles of both citicoline-loaded and unloaded gels were examined. First of all, 1 g of CMC and 1 g of citicoline-loaded CMC gel were frozen at -80°C and subsequently lyophilized at -50°C. The dry weights of the gels were recorded. Following this, 1 mL of PBS was added to each gel, incubated for 1 hour, at 37°C, and centrifuged at 10000 rpm. The supernatant was discarded, and the gels were re-weighed to obtain their wet weights. The swelling ratios were then calculated using following equation : swelling rate (%) = dry weight (g) / wet weight (g) × 100.

Subsequently, 1 mL of PBS was added to the gels again, which were incubated at 37°C. The gels were centrifuged at 10000 rpm on specified days (1, 3, 5, and 9 days). After centrifugation, the supernatant was removed, and the gels were weighed once more. The degradation profiles of the gels were assessed using following equation : degradation rate (%) = wet weight (g) / weight on certain days × 100[7].

The rheological behavior of the gel was analyzed using a rheometer (Malvern Panalytical, Malvern, UK). A 2 cm flat, parallel plate geometry was employed, with a gap of 1 mm between the plates. Zero gap calibration was conducted before each measurement, and all experiments were performed at a temperature of 37°C. The storage modulus (G') and loss modulus (G") of the gels were measured using a strain sweep test, conducted at a frequency of 1 rad/s over a strain range of 0.01% to 100%. Additionally, the viscosity of the gel was determined at a constant frequency of 1 rad/s while varying the shear rate from 0.1 to 100 s^{-1} [25]. The citicoline release study was conducted using a UV-Vis spectrophotometer at a wavelength of 272 nm[31]. Citicoline was dissolved at various concentrations in physiological serum (SF, pH 7.4), and a calibration curve was established. Approximately 1 g of gel, both with and without citicoline, was placed in 5 mL of SF and incubated at 37°C. Samples were collected on specified days, and the amount of released citicoline was measured (n=3)[42]. After incubation, the gels were centrifuged at 10000 rpm, and the citicoline concentration in the supernatant was determined using the UV-Vis spectrophotometer. This value was then used to calculate the loading efficiency of citicoline using following equation : loading efficiency (%) = amount of wash solution / initial amount × 100.

Study groups and surgical procedure

Before the *in vivo* experiment, an a priori sample size calculation was performed using G*Power software (version 3.1.9.7; Heinrich-Heine-Universitat Düsseldorf, Düsseldorf, Germany). The calculation was based on the primary histomorphometric outcome, regenerated axon diameter, and on effect size estimates derived from the experimental sciatic nerve injury study by Sakar et al.[34] in which poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) and human mesenchymal stem cells (MSCs) were evaluated. Using their axon diameter data, an effect size of f=2.16 was obtained for a fixed-effects, one-way omnibus analysis of variance with three groups. With a two-sided α error probability of 0.001 and a desired power (1–β) of 0.9999, the required total sample size was calculated as n=21 animals. To compensate for an anticipated dropout rate of approximately 10%, the planned sample size was increased to 24 rats, with eight animals allocated to each experimental group.

A total of 24 rats were randomly allocated to three experimental groups in a 1 : 1 : 1 ratio (n=8 per group) using a simple randomization procedure to ensure an unbiased and unpredictable assignment of animals. In all animals, a 10 mm segment of the right sciatic nerve was excised and replaced with either PHBA + citicoline (group S), PHBA alone (group T), or an autograft (group O, control).

All surgical procedures were performed under a surgical microscope at the Microsurgery Laboratory (Ordu, Turkey). The rats were placed in the prone position on the operating table. One hour prior to surgery, each animal received an intramuscular injection of Cephazolin sodium (10 mg/kg) for infection prophylaxis. Anesthesia was induced via intraperitoneal injec-

tion of ketamine (1 mg/kg, Ketalar; Pfizer, New York, NY, USA) and xylazine (1 mg/kg, Alfazyne; Alfasan, Woerden, The Netherlands).

Following exposure of the right sciatic nerve, a 10 mm nerve defect was created. In group O, the excised nerve segment was used for autologous repair (Fig. 2A). In group T, the defect was bridged using a PHB/ALG tubular graft (Fig. 2B), whereas in group S, the gap was repaired with a PHB/ALG tubular graft filled with citicoline gel (Fig. 2C). Epineurial anastomosis was performed using 10-0 non-absorbable polyamide sutures. Skin and subcutaneous tissues were closed with 3-0 silk sutures.

All operations were conducted under aseptic conditions using sterile microsurgical instruments and performed by the same experienced surgeon.

Tissue processing and histological evaluation

General anaesthesia was induced intraperitoneally with ketamine (1 mg/kg, Ketalar; Pfizer) and xylazine (1 mg/kg, Alfazyne; Alfasan), followed by intracardiac perfusion with 2% paraformaldehyde in 0.1 mol/L cacodylate buffer at pH 7.4 until the lower extremities were fixed. Euthanasia was achieved by intracardiac injection of 2% paraformaldehyde, following deep anesthesia and loss of reflexes. After perfusion, the right sciatic nerve was carefully dissected. For histopathological evaluation, sciatic nerve tissues were fixed in 10% formaldehyde and embedded in paraffin following routine tissue processing protocols. Sections of 4 μm thickness were obtained from the paraffin blocks. These sections were stained with hematoxylin and eosin and Masson's Trichrome. The following parameters were evaluated under light microscopy : edema, degeneration, necrosis, number of blood vessels, inflammatory cell infiltration in the nerve adventitia, and regenerated axon diameters across the groups. Histopathological data are presented in Tables 1 and 2.

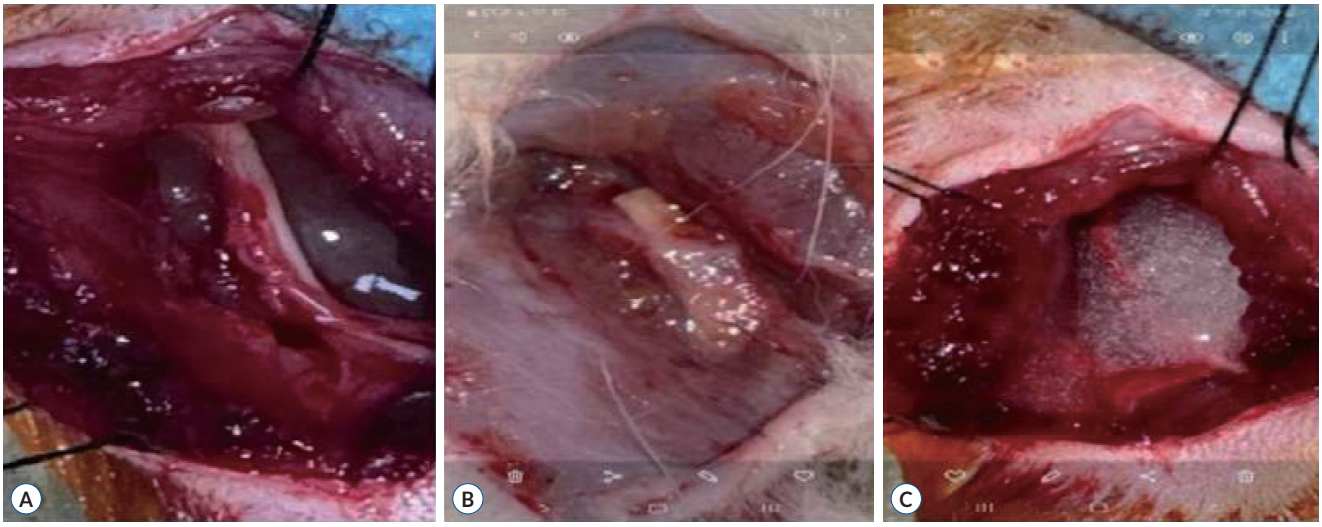


Fig. 2. Surgical procedure and macroscopic view of nerve repair. Group O, the gap was repaired with the excised nerve (autograft) (A). Group T, the gap was repaired with a tubular graft of PHB/ALG structure (B). Group S, the gap was repaired with a tubular graft made of PHB/ALG and filled with citicoline gel (C). PHB : poly(3-hydroxybutyrate), ALG : alginate.

Table 1. Histopathological comparison of nerve sheath degeneration, edema, inflammatory cell infiltration, necrosis and number of blood vessels in the groups

	PHBA graft	Autograft	Citicoline loaded PHBA graft
Nerve sheath degeneration	1.25±0.46	0.63±0.74	1.13±0.35
Edema between nerve fibre bundles	1.13±0.35	1.63±0.74	1.38±0.52
Inflammatory cell infiltration around the nerve adventitia	1.63±0.52	1.85±0.74	1.13±0.35
Necrosis	1.13±0.35	0.75±0.89	1.00±0.53
Number of blood vessels	1.62±0.52	2.50±0.53	1.88±0.83

Values are presented as mean±standard deviation. PHBA : poly(3-hydroxybutyrate)/alginate

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Table 2. Regenerated axon diameters of the groups (μm)

Group	Axon diameter (μm)
PHBA graft	690.75±104.9
Autograft	810.88±69.2
Citicoline loaded PHBA graft	835.63±162.1

Values are presented as mean±standard deviation. PHBA : poly(3-hydroxybutyrate)/alginate

Statistical analysis

Descriptive statistics for continuous variables are presented as mean±standard deviation (SD), median (interquartile range, IQR), mean rank, and minimum-maximum values. The normality of the variables was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated with Levene's test. Since the assumptions for parametric testing were not met, comparisons among the three study groups were performed using the non-parametric Kruskal-Wallis test. For *post hoc* analyses, pairwise comparisons were conducted with Dunn's test and Bonferroni correction to adjust for multiple testing (α=0.05/3). These tests were conducted in SPSS version 30 (IBM Corp., Armonk, NY, USA). Categorical histopathological parameters were summarized as absolute counts (n) and percentages (%) for each severity category (none, mild, moderate, severe) within study groups. Given the small sample size and ordinal nature of these scores, group comparisons were carried out using the Fisher-Freeman-Halton extension of Fisher's exact test. R version 4.5.0 within RStudio version 2024.12.1+ 563 (2024; RStudio Team, Boston, MA, USA) was used for both performing Fisher-Freeman-Halton tests and creating graphs showing the distribution of variables. The type I error rate (α) was set at 0.05 (Two-tailed).

RESULTS

Evaluation of tubular grafts in PHB and PHBA structure

PHB and PHBA tubular grafts *in vitro* water retention, degradation, protein adhesion and cell culture studies were studied with six repetitions. Comparison between groups was made with the Tukey test. p-value <0.05 was accepted. The physical properties of the samples were assessed using SEM. Both horizontal and vertical images of the membranes were analyzed. Additionally, the homogeneity of the ALG coating was evaluated (Fig. 1C-F). The results indicated that the diameters of the PHB fibers were approximately 0.91±0.23 μm, and the ALG coating was found to be stable and homogeneous. The water retention (Fig. 1G) and *in vitro* degradation properties (Fig. 1H) of the membranes were measured using a gravimetric method. The findings showed that the PHB membranes swelled by 132.9%±16.6%, while the PHBA membranes exhibited a swelling of 428.4%±49.8%. The significantly higher swelling ratio of the PHBA membranes compared to the PHB membranes is believed to be attributable to the ALG coating. Upon examining the degradation profiles, it was observed that PHB membranes did not degrade over a period of 6 weeks (p>0.05). In contrast, PHBA membranes initially swelled and began to lose weight by the fifth week (p<0.05). This indicates that the ALG coating remained stable on the PHB membranes for at least 5 weeks. The mechanical properties of the membranes were analyzed using stress-strain curves (Fig. 1I). The brittleness of biomaterials produced with PHB limits their range of applications. To address this limitation, researchers have developed and characterized blend or composite materials[10,19]. Upon examining Fig. 1I, it is evident that the ALG coating increases the elongation of PHB membranes from 30% to 32%. Additionally, it raises the stress value at break from 152 kPa to 161 kPa. The surface properties of materials significantly influence protein adsorption and cell adhesion. While the hydrophilicity of ALG may be advantageous in situations where cell adhesion is undesirable, it poses challenges in tissue engineering applications where cell adhesion is essential[33]. In the presented study, the adhesion of human serum albumin to PHB and PHBA membranes was examined (Fig. 3A). The results showed that 42.7±3.9 μg/mL of protein adhered to the surface of PHB membranes, while only 28.3±2.7 μg/mL of protein adhered to the ALG-modified surface. This indicates that ALG modification reduced human serum albumin adhesion by approximately 66% (p<0.05). Cell adhesion studies produced results consistent with those observed for protein adhesion (Fig. 3B). On the first day of the study, it was found that the number of cells adhering to PHBA membranes was approximately 64% lower than that on PHB membranes (p<0.05). By the seventh day, it was noted that 50% fewer cells had proliferated on the surface of PHBA membranes compared to PHB membranes (p<0.05)[9]. Oxidation products of biomaterials can contribute to toxicity and mutagenicity. Among the nucleobases, guanosine is the most susceptible to oxidation, leading to the formation of its oxidation product,

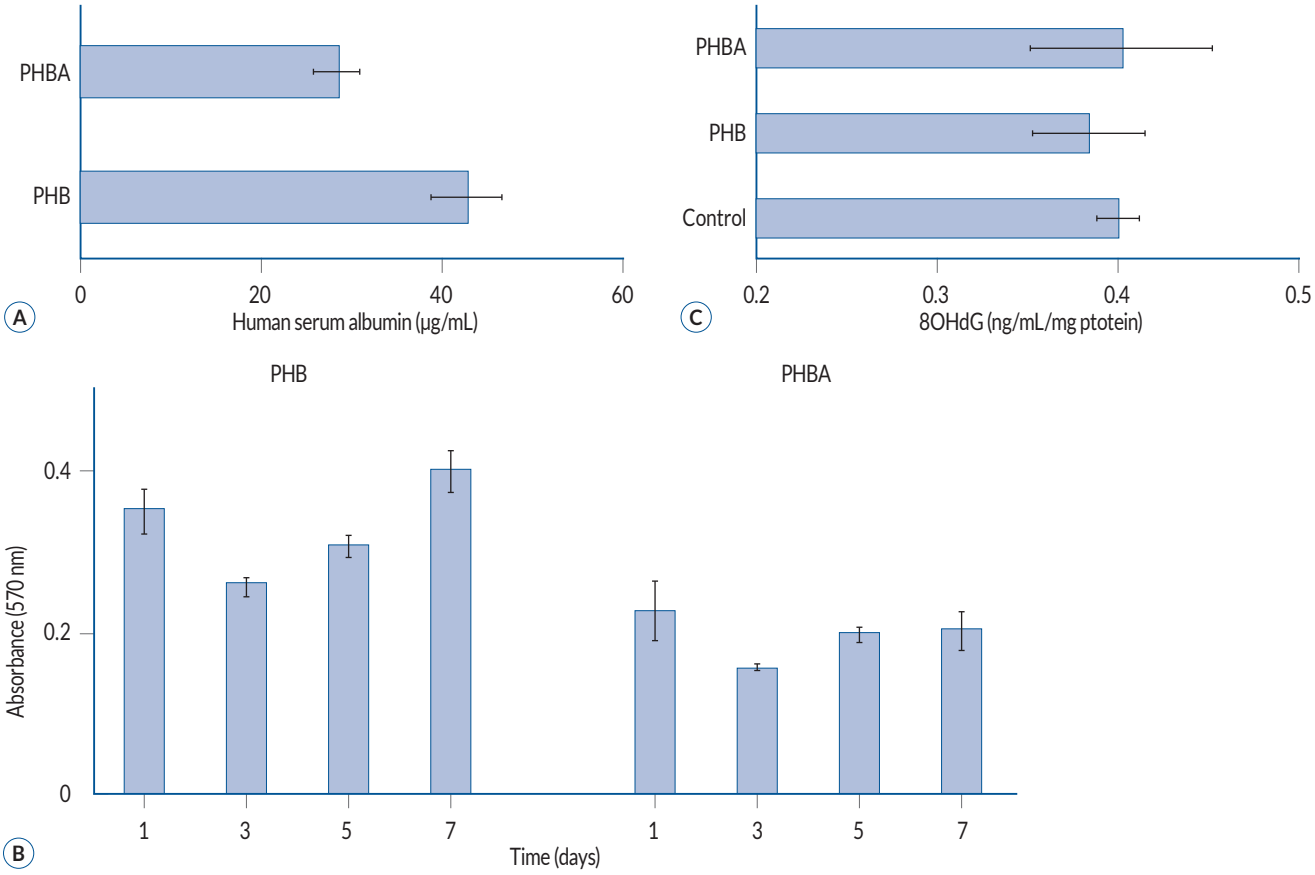


Fig. 3. *In vitro* biocompatibility of membranes. A : Protein adhesion properties of the PHB and PHBA membrane was determined. n=6, $p<0.05$. B : L929, fibroblast cells were cultured on the PHB and PHBA membranes. Cell density was determined by MTT test. n=6, $p<0.05$. C : The effect of PHB and PHBA membranes on DNA oxidation was determined by measuring the amount of 8OHdG. n=6, $p<0.05$. PHBA : poly(3-hydroxybutyrate)/alginate, PHB : poly(3-hydroxybutyrate), 8OHdG : 8-hydroxy-2'-deoxyguanosine, MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

8OHdG[40]. In this study, the oxidation properties of the produced membranes were assessed using DNA as a substrate (Fig. 3C). The results indicated that the levels of 8OHdG in samples containing PHB and PHBA did not significantly increase compared to the control group ($p<0.05$).

Evaluation of citicoline gel

Swelling, degradation, and release studies were conducted with three repetitions. After assessing the normal distribution and homogeneity of the results, variance analysis was performed. Subsequently, group comparisons were made using Tukey's HSD test. A p-value of less than 0.05 was considered statistically significant. The swelling degradation profiles of the gels were determined using the gravimetric method (Fig. 4A). The results indicated that the CMC gel swelled by 158%±5.03%, while the citicoline-loaded gels swelled by 189.8%±14.7%. It was observed that the citicoline-loaded gels retained more water than the CMC gel, which was statistically significant ($p<0.05$). When examining the degradation profiles of the gels, both types lost weight over a period of 9 days, but the degradation rates were not statistically significant ($p>0.05$)[32]. The viscosity values and the loss and storage moduli at increasing frequencies were measured to assess the rheological properties of the gel. The results showed that the viscosities of the gels decreased as the shear rate increased. At a frequency of 1 s^{-1}, the shear rate of the citicoline-containing gel was measured to be 66.3 Pa·s, while the CMC gel had a shear rate of 54.2 Pa·s (Fig. 4B). Viscosity measurements demonstrated that the gels exhibited pseudoplastic properties, and the addition of citicoline slightly increased the viscosity. The G' values for both the CMC (Fig. 4C) and CMC-citicoline gels (Fig. 4D) were found to be higher than the G" values at increasing frequencies, indicating that the

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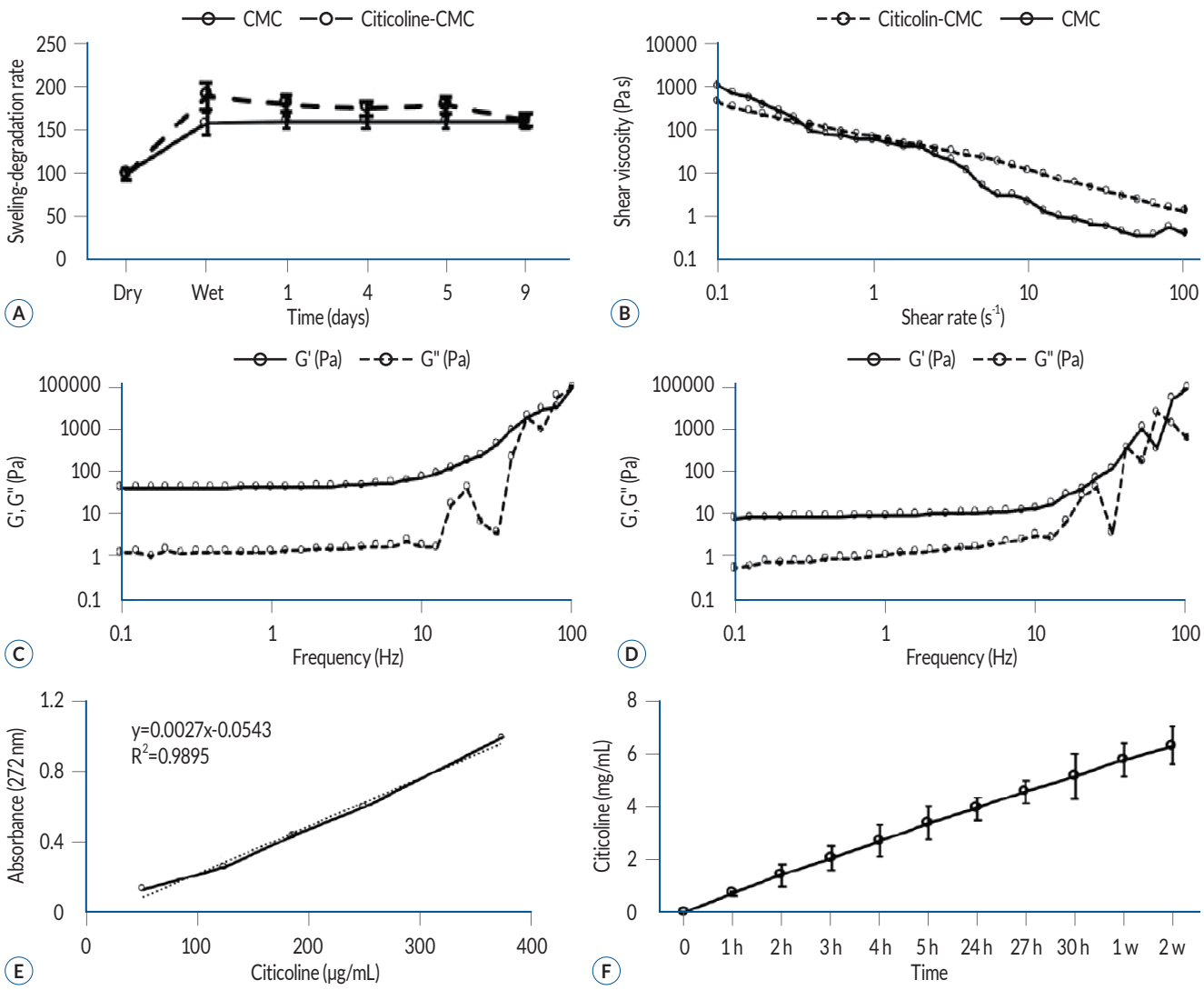


Fig. 4. Characterization and drug release profile of citicoline-loaded CMC gel. A : Swelling and degradation profiles of CMC and citicoline-loaded CMC gels were determined by gravimetric method. n=3, $p<0.05$. B : Viscosity of CMC and citicoline loaded CMC gels were determined. Storage and loss moduli of the CMC gel (C) and citicoline loaded CMC gel (D) were determined at increasing frequency. E : The maximum UV-Vis absorbance of citicoline was determined as 272 nm and a calibration curve was created. Then, the released amount of citicoline was determined on the specified days. The cumulative release profile of citicoline was determined (F). n=3, $p<0.05$. CMC : carboxymethylcellulose, citicoline : cytidine 5′-diphosphocholine, G' : storage modulus, G'' : loose modulus, h : hour(s), w : week(s), UV-Vis : ultraviolet-visible spectrophotometry.

gels-maintained stability under increasing stress. However, the gels began to lose stability at a frequency of approximately 10 Hz. The stress associated with the loss of stability was not related to the addition of citicoline. A calibration curve for citicoline was created based on the determined concentrations (Fig. 4E), yielding a coefficient of determination of 0.989. The equation of the curve was identified as y = 0.0027x - 0.0543. The CMC gel without citicoline served as a blank in this study. The release profiles of the CMC-citicoline gels were evaluated over a 2-week period (Fig. 4F). The release results indicated that the cumulative amount of released citicoline increased over time ($p<0.05$). Initially, the release was rapid, followed by a relatively constant amount of released citicoline[23].

Histopatological evaluation

All rats survived throughout the experimental period. Superficial wound infection was observed in two rats in group T and one rat in group S and treated with local dressing. PHBA

grafts did not deteriorate *in vivo*, but moderate tissue reaction was observed. A thin capsule structure consisting of mononuclear phagocytic cells, lymphocytes and fibroblasts was observed on the outer surface of the grafts. Photographs of histological examinations are given in Fig. 5A-F.

Table 3 and Fig. 5G present the descriptive statistics for sciatic

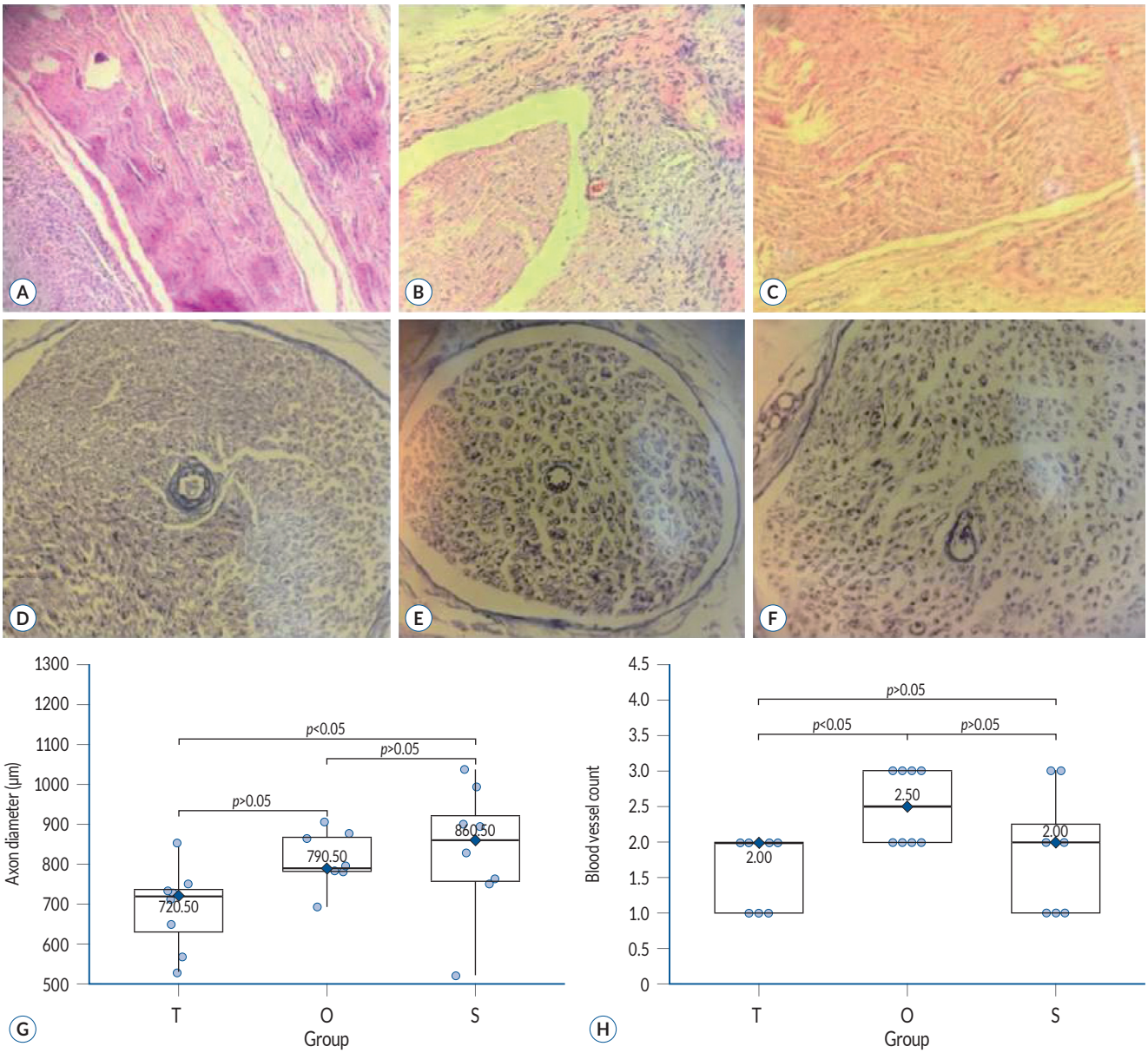
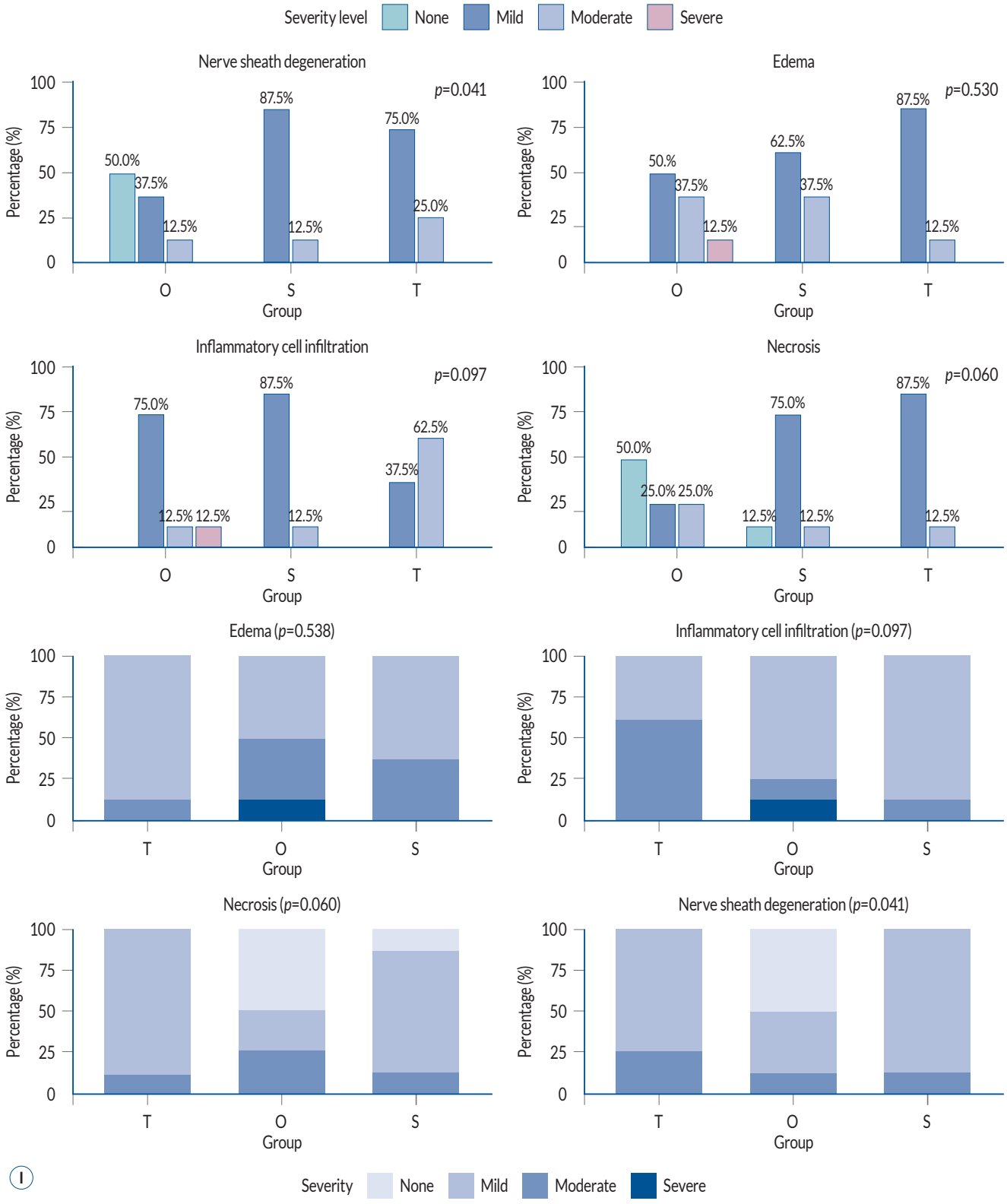


Fig. 5. Histological and morphometric analysis of regenerated sciatic nerves. Histopathological images of autograft (A), citicoline containing PHBA (B) and PHBA (C) groups in terms of blood vessel, nerve sheath degeneration, inflammatory cell, edema and necrosis parameters (hematoxylin and eosin, ×200). Histological images showing regenerated axon diameters of autograft (D), PHBA containing citicoline (E) and PHBA (F) groups (Masson Trichrome, ×200). G : Box-plot illustrating the distribution of axon diameter (μm) across the three study groups. The central line inside each box represents the median (Q2); box edges denote the first (Q1) and third (Q3) quartiles (IQR); whiskers extend to the minimum and maximum values; blue dots represent individual observations. H : Box-plot illustrates the distribution of blood vessel counts across the three study groups. The central line inside each box represents the median (Q2); box edges denote the first (Q1) and third (Q3) quartiles (IQR); whiskers extend to the minimum and maximum values; blue dots represent individual observations. The *p*-values shown in (G and H) represent Dunn-Bonferroni-adjusted *post hoc* pairwise comparisons performed after a significant Kruskal-Wallis test. I : Severity distributions (%) of nerve sheath degeneration, edema, inflammatory cell infiltration, and necrosis by the groups, and statistical comparisons. T : PHBA graft group, O : autograft group, S : citicoline-loaded PHBA graft group, PHB : poly(3-hydroxybutyrate), PHBA : poly(3-hydroxybutyrate)/alginate, IQR : interquartile range.

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(I)

Fig. 5. Continued.

Table 3. Descriptive statistics of sciatic nerve axon diameter (μm) by the groups

Group	Mean±SD	Median (IQR)	Mean rank	Min.-Max.	*p*-value
PHBA graft (n=8)	690.8±104.9	720.5 (160)	7.1 a	530.0-854.0	0.028
Autograft (n=8)	810.9±69.2	790.5 (94)	14.8 ab	693.0-909.0	
Citicoline loaded PHBA graft (n=8)	835.6±162.1	860.5 (216)	15.7 a	522.0-1038.0	

Overall comparison : Kruskal-Wallis test, p=0.028. *Post-hoc* analysis : Dunn-Bonferroni test : Groups sharing at least one common letter are not significantly different (p>0.05). SD : standard deviation, IQR : interquartile range, PHBA : poly(3-hydroxybutyrate)/alginate, Min. : minimum, Max. : maximum

Table 4. Descriptive statistics of blood vessel count by the groups

Group	Mean±SD	Median (IQR)	Mean rank	Min.-Max.	*p*-value
PHBA graft (n=8)	1.63±0.52	2.0 (1.0)	9.13 a	1-2	0.044
Autograft (n=8)	2.50±0.54	2.5 (1.0)	17.00 a	2-3	
Citicoline loaded PHBA graft (n=8)	1.88±0.84	2.0 (1.75)	11.38 ab	1-3	

Overall comparison : Kruskal-Wallis test, p=0.028. *Post-hoc* analysis : Dunn-Bonferroni test : Groups sharing at least one common letter are not significantly different (p>0.05). SD : standard deviation, IQR : interquartile range, PHBA : poly(3-hydroxybutyrate)/alginate, Min. : minimum, Max. : maximum

nerve axon diameters (μm) in groups. Each group included eight samples (n=8), with mean±SD values of 690.8±104.9 μm (median, 720.5; IQR, 160) in PHBA group, 810.9±69.2 μm (median, 790.5; IQR, 94) in autograft group, and 835.6±162.1 μm (median, 860.5; IQR, 216) in citicoline loaded PHBA group. The observed ranges spanned 530–854 μm (PHBA group), 693–909 μm (autograft group), and 522–1038 μm (citicoline loaded PHBA group). A Kruskal-Wallis test revealed a statistically significant difference among the three groups (p=0.028). In post-hoc pairwise comparisons, citicoline loaded PHBA group (mean rank, 15.7) exhibited significantly larger axon diameters than PHBA group (mean rank, 7.1) (p<0.05), whereas autograft group (mean rank, 14.8) did not differ significantly from either PHBA group or citicoline loaded PHBA group (p>0.05).

Table 4 and Fig. 5H present the descriptive statistics for blood vessel counts in the groups. Each group included eight samples (n=8), with mean±SD values of 1.63±0.52 (median, 2.0; IQR, 1.0; range, 1–2) in PHBA group, 2.50±0.54 (median, 2.5; IQR, 1.0; range, 2–3) in autograft group, and 1.88±0.84 (median, 2.0; IQR, 1.75; range, 1–3) in citicoline loaded PHBA group. A Kruskal-Wallis test revealed a statistically significant difference among the three groups (p=0.044). In Dunn's *post hoc* pairwise comparisons, autograft group (mean rank, 17.00) exhibited a significantly higher vessel count than PHBA group (mean rank, 9.13) (p<0.05), whereas citicoline loaded PHBA group (mean rank, 11.38) did not differ significantly from either PHBA group or autograft group (p>0.05).

The distributions and statistical differences of the histopathological characteristics of the study groups, such as nerve sheath degeneration, edema, inflammatory cell infiltration, and necrosis, are summarized in Table 5 and Fig. 5I. In histopathological evaluations, a statistically significant difference was observed among the groups in terms of nerve sheath degeneration (p=0.041). In the PHBA group, degeneration was present in all samples, with 75% classified as mild and 25.0% as moderate. In contrast, no degeneration was observed in 50% of the samples in the autograft group, while 37.5% showed mild and 12.5% moderate degeneration. Notably, degeneration was present in all samples in the citicoline loaded PHBA group as well; however, 87.5% of these were mild and 12.5% were moderate. Although the 100.0% incidence of degeneration in the citicoline loaded PHBA group (which received citicoline) is remarkable, the predominance of mild severity suggests that citicoline may partially preserve myelin integrity and limit the degenerative process.

No statistically significant differences were detected among the groups regarding edema (p=0.538). However, distributional analysis revealed that mild and moderate edema were present in 87.5% and 12.5% of PHBA group samples, respectively. In the autograft group, 50.0% showed mild, 37.5% moderate, and 12.5% severe edema, while the citicoline loaded PHBA group exhibited 62.5% mild and 37.5% moderate edema. No statistically significant difference was found among the groups in terms of inflammatory cell infiltration (p=0.097). Moderate infiltration was predominant in the PHBA group (62.5%), whereas mild infiltration was observed in 75.0% of autograft group

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Table 5. Severity distributions (%) of nerve sheath degeneration, edema, inflammatory cell infiltration, and necrosis by groups, and statistical comparisons

	Group			*p*-value*
	PHBA graft	Autograft	Citicoline loaded PHBA graft	
Nerve sheath degeneration				0.041
None	0 (0.0)	4 (50.0)	0 (0.0)	
Mild	6 (75.0)	3 (37.5)	7 (87.5)	
Moderate	2 (25.0)	1 (12.5)	1 (12.5)	
Severe	0 (0.0)	0 (0.0)	0 (0.0)	
Total	8 (100.0)	8 (100.0)	8 (100.0)	
Edema				0.538
None	0 (0.0)	0 (0.0)	0 (0.0)	
Mild	7 (87.5)	4 (50.0)	5 (62.5)	
Moderate	1 (12.5)	3 (37.5)	3 (37.5)	
Severe	0 (0.0)	1 (12.5)	0 (0.0)	
Total	8 (100.0)	8 (100.0)	8 (100.0)	
Inflammatory cell infiltration				0.097
None	0 (0.0)	0 (0.0)	0 (0.0)	
Mild	3 (37.5)	6 (75.0)	7 (87.5)	
Moderate	5 (62.5)	1 (12.5)	1 (12.5)	
Severe	0 (0.0)	1 (12.5)	0 (0.0)	
Total	8 (100.0)	8 (100.0)	8 (100.0)	
Necrosis				0.060
None	0 (0.0)	4 (50.0)	1 (12.5)	
Mild	7 (87.5)	2 (25.0)	6 (75.0)	
Moderate	1 (12.5)	2 (25.0)	1 (12.5)	
Severe	0 (0.0)	0 (0.0)	0 (0.0)	
Total	8 (100.0)	8 (100.0)	8 (100.0)	

Values are presented as number (%). *Fisher-Freeman-Halton test. PHBA : poly(3-hydroxybutyrate)/alginate

and 87.5% of citicoline loaded group samples. Similarly, necrosis did not differ significantly among the groups (p=0.060). In the PHBA group, 87.5% of the samples showed mild and 12.5% moderate necrosis. In the citicoline loaded PHBA group, necrosis was absent in 50% of samples, with the remaining 50.0% equally distributed between mild (25.0%) and moderate (25.0%) severity. In the citicoline loaded PHBA group, 75.0% of samples exhibited mild necrosis, 12.5% moderate, and 12.5% showed no necrosis.

DISCUSSION

It is well established that the brittleness of biomaterials produced with PHB limits their applicability in practical settings. To overcome this limitation, researchers have developed and characterized composite materials[10,19]. In this study, PHB membranes were homogeneously coated with ALG using a 1% ALG solution prepared in distilled water to enhance the mechanical properties of PHB. This modification aimed not only to improve scaffold strength but also to reduce inflammation and tissue scarring. PHBA grafts maintained their structural integrity and provided sufficient space for nerve regeneration by the sixteenth postoperative week.

The hydrophilic nature of ALG has been investigated in the literature and is considered advantageous in applications where cell adhesion is undesired[33]. This property is known to reduce cell adherence. In our study, ALG modification led to a 66% re-

duction in human serum albumin adhesion (p<0.05). Similarly, cell proliferation was reduced by 64% on day 1 and by 50% on day 7 (p<0.05)[9]. These findings suggest that the PHBA graft may attenuate inflammation and scar formation, and that the ALG coating contributes to preserving the anatomical integrity of the graft.

Biomaterial oxidation products can induce toxicity and mutagenicity, which may adversely affect wound healing[27]. Therefore, the oxidation properties of the produced membranes were evaluated using DNA as a substrate. The results showed no significant increase in 8OHdG levels in PHB or PHBA groups compared to controls (p<0.05), indicating that PHBA grafts are neither mutagenic nor toxic, supporting their potential safety in future clinical use.

In our study, it was aimed to further increase axonal regeneration by local addition of citicoline molecule in a releasable gel structure to the medium. Citicoline-loaded gels exhibited significantly greater water retention than CMC gels (p<0.05). Although both gel types degraded over nine days, the degradation rates were not significantly different (p>0.05)[32]. Release studies demonstrated a cumulative increase in citicoline levels over time, beginning with a rapid release followed by a sustained phase (p<0.05)[23]. These findings confirm that citicoline was consistently released into the microenvironment, likely maintaining its presence at therapeutic levels in the injury zone and contributing to axonal regeneration.

The groups were also compared histopathologically in terms of regenerated axon diameters, number of blood vessels, nerve sheath degeneration, edema, inflammatory cell infiltration and necrosis as in the literature[5,17]. The citicoline-loaded PHBA group yielded axon diameter results comparable to those of the autograft group (p=0.028). The distribution and statistical differences of the histopathological features of the study groups are summarised in Table 5 and Fig. 5I.

In a prior experimental study, it was shown that capillary formation around conduit channels positively influences axonal regeneration[16]. Therefore, blood vessel counts were analyzed histopathologically. The best results were observed in the autograft group. Kruskal-Wallis test revealed a statistically significant difference between the three groups (p=0.044). In Dunn's *post hoc* pairwise comparisons, the autograft group (mean rank, 17.00) exhibited significantly higher vessel count than the PHBA group (mean rank, 9.13) (p<0.05), whereas citicoline-loaded PHBA (mean rank, 11.38) was not significantly different in the PHBA or autograft groups (p>0.05).

A statistically significant difference was also observed in nerve sheath degeneration (p=0.041). No degeneration was noted in 50% of autograft samples, whereas 37.5% showed mild and 12.5% moderate degeneration. In contrast, degeneration was present in all citicoline-loaded PHBA samples, though predominantly mild (87.5%) and moderate (12.5%). The predominance of mild severity of nerve sheath degeneration in the citicoline-loaded PHBA group suggests that citicoline may partially protect myelin integrity and limit the degenerative process. The absence of degeneration in almost half of the samples in the autograft group highlights the continued histological superiority of autologous tissue due to its appropriate immunological and cellular compatibility.

No statistically significant differences were observed among groups for edema (p=0.538). These findings suggest that edema is likely caused by common pathophysiological mechanisms such as graft-related surgical trauma, increased vascular permeability and local inflammation, and that citicoline administration alone does not significantly modulate this process.

Inflammatory cell infiltration also did not differ significantly (p=0.097), though milder infiltration in the citicoline-loaded PHBA group indicates a potentially reduced immune response. Conversely, more pronounced infiltration in the PHBA group may reflect a reaction to the synthetic scaffold. Similarly, necrosis showed no significant group differences (p=0.060), though lower severity in the citicoline-loaded group suggests that citicoline may mitigate necrotic damage.

Citicoline is a molecule that affects choline metabolism, provides membrane stabilisation and has anti-inflammatory properties. The UV-Vis-based release profile in this study demonstrated an initial burst release followed by a sustained release phase (p<0.05), indicating prolonged local bioavailability. Citicoline is known to reduce necrosis by stabilizing membranes, enhancing antioxidant capacity, and preserving mitochondrial function. Previous studies have reported that citicoline-containing agents support retinal ganglion cell survival in optic neuropathies and may exert neuromodulatory and neuroprotective effects against multiple toxic stimuli[13]. Higher recovery rates have been seen in patients taking citicoline after a stroke. It has been stated that citicoline, a chemical compound involved in the synthesis of cell membranes, is a promising drug in accelerating nerve regeneration[18].

In our study, citicoline-loaded PHBA grafts achieved regen-

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erated axon diameters comparable to those observed in the autograft group.

Descriptive statistics for sciatic nerve axon diameters (μm) in all groups are presented in Table 3 and Fig. 5G. Kruskal-Wallis test revealed a statistically significant difference between the three groups (p=0.028). In *post-hoc* pairwise comparisons, the citicoline-loaded PHBA group (mean rank, 15.7) exhibited significantly larger axon diameters than the PHBA group (mean rank, 7.1) (p<0.05), while the autograft group (mean rank, 14.8) was not significantly different from the PHBA or citicoline-loaded PHBA group (p>0.05).

Overall, the distribution of histopathological parameters suggests that citicoline administration has a protective potential, especially in modulating inflammation and cell death. However, the superior histological integrity observed in the autograft group reinforces the idea that autologous nerve grafts continue to represent the gold standard in nerve repair. While citicoline-assisted biomaterial approaches may offer potential advantages in nerve regeneration, further molecular and functional studies are required to confirm these effects.

It has been shown that repair with neural scaffolds after peripheral nerve injury may contribute to nerve regeneration by causing MSCs to differentiate to form nerve cells[24]. However, synthetic polymers often suffer from inadequate biodegradability and suboptimal biological performance[35]. To address this limitation, combining PHB and hydroxyapatite frameworks with ALG hydrogel has been shown to improve graft mechanical strength significantly[41].

The duration of our experimental study was set at 4 months. According to the literature, axonal regeneration typically begins around the second month following injury. It is well-established that extending the observation period may further enhance axonal regeneration and nerve healing. The key factors influencing the outcome of reconstructive surgery after peripheral nerve injury include the length of the nerve gap, the type of injury, the time elapsed since the injury, and the length and structural properties of the autograft or synthetic conduit used. Given that previous studies have consistently reported optimal outcomes in nerve reconstructions involving gaps shorter than 3 cm—and significantly poorer outcomes in defects exceeding this threshold—we limited the nerve gap to 1 cm in this study to ensure reliable and reproducible results[4,8].

In our study, we found that although citicoline increased the number of vessels, this ratio was insufficient when compared to autograft. We believe that this result partially negatively affected the potential for functional improvement. In a previous experimental study, an inside-out vein graft was employed to facilitate functional recovery of the sciatic nerve following injury. This approach resulted in a significantly greater number of regenerating nerve fibers in the vein graft group compared to the control group[28]. It has been indicated that citicoline exhibits neuroregenerative effects in both the subacute and chronic stages of stroke in cerebrovascular disease by activating angiogenesis and increasing neurotransmitter metabolism[26]. High-dose citicoline may help with flap survival after trauma. It has also been suggested that citicoline treatment after flap surgery may reduce cellular inflammation, oxidative damage and angiogenesis[11].

It has been demonstrated that citicoline treatment significantly increases the number of active microvessels in the ischaemic area 21 days after infarction. For the first time, both *in vivo* and *in vitro* models have been used to demonstrate citicoline's vascular protective and pro-angiogenic effects. Therefore, it has been emphasised that the beneficial effects following chronic treatment may be partially attributable to revascularisation[21]. Experimental studies in rodents and dogs have confirmed that citicoline has no acute or chronic toxicity. No deaths were observed after a single oral dose of 2000 mg/kg citicoline[36]. In light of the information available in the literature, we believe that higher doses and longer-term citicoline support are needed to achieve better clinical outcomes and to serve as an alternative to autografts.

The *in vivo* degradation profile of the graft has not been evaluated in the presented study. However, there are numerous studies in the literature that address this issue. Freier et al.[12] produced a PHB film for abdominal surgery. Researchers developed a PHB-based patch for repairing intestinal defects. This graft in the PHB structure showed resistance to intestinal secretions and remained intact for a sufficiently long period to allow complete healing of the defect. Twenty-six weeks after placing the patch in the intestines of Wistar rats, they observed that only a trace amount of PHB film remained at the surgical site[12]. Gogolewski et al.[14] implanted PHB and PHBV (poly(3-hydroxybutyrate-co-3-hydroxyvalerate)) subcutaneously in mice. After 26 weeks of surgery, they reported a deterioration rate of 15-43 per cent for both PHB and PHBV[14]. These results indicate that tubular grafts with a PHBA structure can remain in the tissue for a sufficient period following surgical repair after

peripheral nerve damage.

In future studies, the incorporation of MSCs into citicoline-loaded PHBA scaffolds could offer a synergistic approach. Given the encouraging biological performance of the PHBA structure observed in this study, these bioengineered combinations may form the basis of future therapeutic strategies.

CONCLUSION

In this experimental sciatic nerve injury model, it was shown that PHBA-based tubular graft with oriented nanofibre surfaces containing citicoline is non-toxic, can be produced in desired size and length and can partially support axonal regeneration. Although the PHBA graft containing citicoline is not as effective as autograft, the results obtained are promising. This bioengineering-based approach may be a candidate for clinical use in cases where autologous graft cannot be applied.

Limitations

In our study, the results were evaluated histopathologically. However, functional outcome assessment (Sciatic function index, electrophysiological tests) was not performed. The absence of functional outcomes is among the limitations of this study.

AUTHORS' DECLARATION

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

Informed consent

This type of study does not require informed consent.

Author contributions

Conceptualization : HÖ, AY, MH; Data curation : MAÇ, MD, EE, SÖ; Formal analysis : YKA; Methodology : VN, MH; Project administration : HÖ, AY, MH; Visualization : HÖ, MH, AY; Writing - original draft : HÖ; Writing - review & editing : HÖ, MH

Data sharing

None

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ORCID

Hıdır Özer	https://orcid.org/0000-0002-1017-2389
Ali Yılmaz	https://orcid.org/0000-0001-5378-4409
Mürüvvet Akçay Çelik	https://orcid.org/0000-0002-0335-4045
Yeliz Kaşko Arıcı	https://orcid.org/0000-0001-6820-0381
Vugar Nabi	https://orcid.org/0000-0002-2766-5083
Ebru Erdal	https://orcid.org/0000-0001-5384-5961
Songül Özay	https://orcid.org/0009-0002-3160-3587
Murat Demirbilek	https://orcid.org/0000-0001-6990-5607
Mehdi Hekimoğlu	https://orcid.org/0000-0002-1182-2216

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