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In vivo application of poly-3-hydroxyoctanoate as peripheral nerve graft

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Abstract: Objective: This study aims to investigate the degree of biocompatibility and neuroregeneration of a polymer tube, poly-3-hydroxyoctanoate (PHO) in nerve gap repair. Methods: Forty Wistar Albino male rats were randomized into two groups: autologous nerve gap repair group and PHO tube repair group. In each group, a 10-mm right sciatic nerve defect was created and reconstructed accordingly. Neuroregeneration was studied by sciatic function index (SFI), electromyography, and immunohistochemical studies on Days 7, 21, 45 and 60 of implantation. Biocompatibility was analyzed by the capsule formation around the conduit. Biodegradation was analyzed by the molecular weight loss in vivo. Results: Electrophysiological and histomorphometric assessments demonstrated neuroregeneration in both groups over time. In the experimental group, a straight alignment of the Schwann cells parallel to the axons was detected. However, autologous nerve graft seems to have a superior neuroregeneration compared to PHO grafts. Minor biodegradation was observed in PHO conduit at the end of 60 d. Conclusions: Although neuroregeneration is detected in PHO grafts with minor degradation in 60 d, autologous nerve graft is found to be superior in axonal regeneration compared to PHO nerve tube grafts. PHO conduits were found to create minor inflammatory reaction in vivo, resulting in good soft tissue response.

Key words: Axonal spurting, Biodegradable polymer, Neuroregeneration, Nerve grafting
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1 Introduction

Autologous peripheral nerve grafting is the standard method of surgery in peripheral nerve injuries. However, donor site morbidity is an unwanted outcome (Hazari *et al*., 1999; Young *et al*., 2002; Huang and Huang, 2006). This has brought an interest in the usage of alternative replacement methods. Tissue engineering has been used in various systems in vivo and in vitro (Chen and Wu, 2005; Xie *et al*., 2008; Han *et al*., 2010; Liu *et al*., 2011). Among those, tissue-engineered peripheral nerve conduits have become a good alternative to autologous nerve grafts in surgical repair of nerve injuries (Wang *et al*., 2005; Chen *et al*., 2006; Kim *et al*., 2006; Yang Y.M. *et al*., 2007; Yang Y.C. *et al*., 2011). Poly(3-hydroxyalkanoates) (PHAs) are a class of reserve polyesters produced by a large number of bacteria

when subjected to metabolic stress (Foster *et al*., 2005; Hazer and Steinbuchel, 2007; Liu *et al*., 2008; Hazer, 2010; Hazer *et al*., 2012b). Different kinds of PHAs have been introduced, such as polyglycolic acid (PGA) (Waitayawinyu *et al*., 2007) and poly(3-hydroxybutyrate) (PHB) (Bian *et al*., 2009) in grafting peripheral nerve injury.

An ideal biodegradable conduit should allow cell-to-cell communication and subsequent tissue ingrowths during the regenerative processes and maintain its chemical structure to some degree (Brunelli *et al*., 1994; Terenghi, 1999; Stang *et al*., 2009; Raimondo *et al*., 2011). For these properties to be achieved, a biomaterial should be flexible and biocompatible and also should remain in situ without degradation beyond the period of regeneration (di Summa *et al*., 2011). There have been several studies of PHB conduits and different kinds of PHAs used for peripheral nerve gap repair and all facilitated nerve regeneration (Kakinoki *et al*., 2011; Sakar *et al*., 2011; Yang Y.M. *et al*., 2011).

Poly-3-hydroxyoctanoate (PHO) is a storage product of bacteria, which undergoes hydrolytic degradation and is completely absorbed in 24–30 months (Marois and Zhang, 1999; Kim *et al*., 2007; Wu *et al*., 2009). In vitro biocompatibility of PHO was demonstrated previously by various authors. Sodian *et al*. (2000) constructed a PHO trileaflet heart valve scaffold and covered the scaffold with vascular cells harvested from ovine carotid arteries in vitro. They had demonstrated a growth of endothelial cells on the scaffold surface. Chung *et al*. (2012) investigated the cellular biocompatibilities of PHO and VI-g-PHO via investigating rat ovarian cell attachment and proliferation on the polymer disk. They had concluded that VI-g-PHO polymer disks have superior in vitro biocompatibility compared to PHO polymer disks.

Our research group has already investigated in vivo biocompatibility of a different kind of PHO based on soya bean oily acid and gold-catalyzed poly(3-hydroxyoctanoate-co-3-hydroxy-10-ndecenoate) (PHOU) and reported that both have favorable soft tissue response (Hazer B. *et al*., 2009; Hazer D.B. *et al*., 2009; Hazer and Hazer, 2011). Therefore, biocompatible and biodegradable PHO nerve conduits seem to be good candidates for nerve grafting because of their structural suitability for suturing and causing less inflammation. The aim of this study is to investigate neuroregeneration and the degree of biocompatibility of a polymer tube PHO in nerve gap repair.

2 Materials and methods

In this study, a total of 40 Albino Wistar male rats weighting 250 g were used. All procedures were carried out in compliance with Hacettepe University Ethical Committee, Turkey.

2.1 Preparation of the PHO tubing

PHO is a member of PHAs, which was obtained from *Pseudomonas oleovorans* grown on octanoic acid. Solvent casting of 2.0 g PHO dissolved in 15 ml of $CHCl_3$ solution was used to prepare PHO tubing. A steel wire (length: 15 cm, diameter: 15 mm) was inserted into this solution and taken out via continuous manual swirling to allow evaporation of the solvent. It was left to dry in air and in a vacuum for a day for the final structural composition. This procedure was repeated 15 times until a thick PHO film was formed smoothly on the steel wire. The PHO film formed on the steel wire was washed with methanol, dried in a vacuum for a day. A long polymer tube was peeled off and cut into multiple standard pieces (12.0 mm long, 1.5 mm in diameter, and 1.0 mm in thickness). Then they were sterilized by ethylene oxide gas for 8 h.

2.2 Nerve gap repair

The operation was performed in a similar fashion to that previously described in the literature (di Summa *et al*., 2011). Accordingly, the left sciatic nerve was exposed with a skin incision from the right knee to the hip under surgical microscope (Carl Zeiss, Germany). In the experimental group, a 10-mm nerve gap was created and nerve ends were fixed to the conduit with two epineural sutures (10/0 Prolene, Ethicon, Germany) in such a way that the proximal and distal nerve stumps were inserted 1 mm into the tube to leave a 10-mm gap. In the autograft group, a 10-mm nerve segment was excised, reversed, and resutured on each side by two epineural sutures (di Summa *et al*., 2011; Raimondo *et al*., 2011) Concomitant layers were closed in anatomical order (4/0 Softcat, Braun, Germany, for muscles and fascia layers, and 4/0 Prolene, Ethicon, Germany, for skin) (Fig. 1).

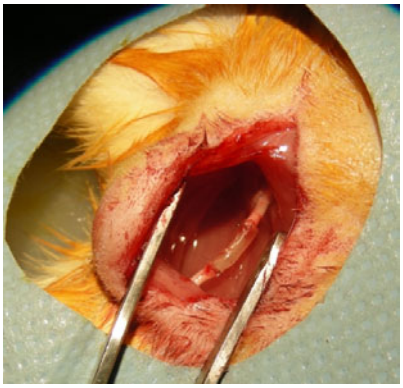

Fig. 1 Demonstration of PHO nerve conduit sutured to sciatic nerve in a rat

2.3 Walking track analysis

All rats were taken to their cages and observed for 60 d under standard environmental conditions. Sciatic functional index (SFI) was used for the evaluation of nerve regeneration on Days 7, 14, 21, 45, and 60, respectively (Chen *et al*., 2007). The animal was placed in a walking pathway, which ends in a darkened cage (Varejão *et al*., 2001; Özmen *et al*., 2002; Mohammadi *et al.*, 2011). The rat's hind feet were dipped in stamp paint, and the animal was permitted to walk down the track, leaving its hind footprints on the paper. Then, paired measurements of the print length (distance between heel and the tip of the third toe), toe spread (equal to the distance between the tip of first and fifth toes), intermediate toe spread (equal to the distance between the tip of second and fourth toes), and the distance to the opposite toe were taken for both the unoperated and experimental sides. The SFI was calculated by the formula proposed by Bain *et al*. (1989). SFI values of each rat were collected and mean and median values were calculated with standard deviations (SDs).

In general, the SFI values close to "0" will demonstrate normal nerve function, whereas "−100" SFI demonstrates total dysfunction. The SFI data was assessed based on the Sham group, and the normal level was considered as "0". The SFI data was gathered as a negative value, and therefore a higher SFI meant the better function of the sciatic nerve.

2.4 Electrophysiological analysis

Electrophysiological studies were performed 21 and 60 d after surgery on five randomly selected rats in each group before tissue harvesting. A commercial electromyography device (Medtronic Keypoint 4) was used for all stimulation and recording procedures. Both legs of the rats in each group were shaved and bipolar platinum stimulating electrodes were placed 10 mm proximal to the proximal coaptation line on the experimental side and at the corresponding location on the opposite, control side. A recording electrode measuring the evoked compound muscle action potential (CMAP) was placed on the belly of gastrocnemius muscle, and a reference electrode was placed at the tendon of the gastrocnemius muscle. A disk electrode (2 mm in diameter) was placed in a superficial muscle layer near the skin as a ground electrode. The onset latency, amplitude, and area under the amplitude of CMAP were measured on both sides for each rat using computer software. All data were gathered from each rat as the ratio of variables of the experimental limb to control limb of the rat (Sariguney *et al*., 2008).

2.5 Graft harvesting and histological sectioning

Five animals from each group were sacrificed at 7, 21, 45, and 60 d postoperatively. The regenerated right sciatic nerve (14 mm length) including the proximal and distal stumps was harvested under operating microscope. These nerve tissue blocks were fixed and embedded in paraffin and ultramicrotome serial cross sections of 5 μm thickness were taken. These sections were taken from five different locations of the whole harvested segment: starting 1 mm distal and 1 mm proximal to the proximal suture on the proximal stump, in the center of the nerve segment (6th mm), and finally, 1 mm distal and 1 mm proximal to the distal suture on the distal stump in order to allow visualization of regenerated fibers entering the distal nerve stump. Hematoxylin-eosin (HE) staining and a panel of antibody markers were employed, comprising anti-neurofilament (pan) (Clone FNP7, Zymed Lab, CA, USA; dilution 1:200), a pan-neuronal marker; S-100 (Zymed Lab, CA, USA; prediluted), an antibody marker for Schwann cells; and finally CD68 (Clone KP1, Zymed Lab, CA, USA; prediluted) for macrophages.

2.6 Immunohistochemical evaluation

The immunohistochemical studies were conducted on the cross-sectioned nerve samples taken

from the harvested regenerated nerves described above. The morphological analysis of the soft tissue response of a new biomaterial was described in our previous reports (Hazer D.B. *et al*., 2011; 2012a). It was analyzed by the capsule formation around the conduit, and the parameters are the capsule thickness, polymorphonuclear cell and giant cell accumulation, and new blood vessel formation within the capsule. These data were assessed from four different standardized areas of the cross sections of the capsule: superior, inferior, left, and right lateral thirds of the capsule around the implant and autograft (Fawcett and Keynes, 1990; Rickett *et al*., 2011). The final value was recorded as the mean value of these measurements.

For the immunohistochemical analysis, five representative fields of the nerve cross-sectional area were determined on each cross-sectioned nerve sample under 400× magnification: superior, inferior, left, and right lateral thirds and central body of the sample. Anti-neurofilaman protein (NFP) staining, S100 staining for Schwann cells, and CD68 staining for macrophages within each fiber were counted in each section described above and mean values were calculated for each group (di Summa *et al*., 2011).

The sciatic nerve diameter in each HE-stained cross section was measured and mean values were detected.

2.7 Statistical analysis

The values collected from electromyography (EMG) recording were evaluated with normalized data. Together with the normalized EMG data, SFI values and immunohistochemical results with capsule thickness were statistically analyzed between and within the group. The comparison between EMG recording data of the control and experimental groups according to changes in days was made by Mann-Whitney *U* test ($p<0.05$ designates statistical significance). The effects of groups and changes in days on EMG data were analyzed by univariate general linear models analysis of variance (ANOVA) ($p<0.05$ designates statistical significance). Among groups, the difference between SFI values was detected again by Mann-Whitney *U* test. Comparison within each group for SFI values with day changes was made by Freidmann test. Immunohistochemical parameters were compared according to changes in days within groups by Friedmann test. The effects of groups and changes in days on immunohistochemical data were analyzed by univariate general linear ANOVA ($p<0.05$, $p<0.01$, and $p<0.001$ designate statistical significance).

3 Results

3.1 Macroscopic results

The rats in each group were healthy and there was no necrosis, abscess, or wound infection neighboring the implants. Throughout the implantation period, antibiotics were not used. In the control group, there was prominent fibrosis around the autograft in an increasing order from Days 7 to 60. In the PHO group, fibrosis around the conduit was less when compared to the autograft and the conduit could easily be dissected from the surrounding tissue even on Day 60 (Fig. 2).

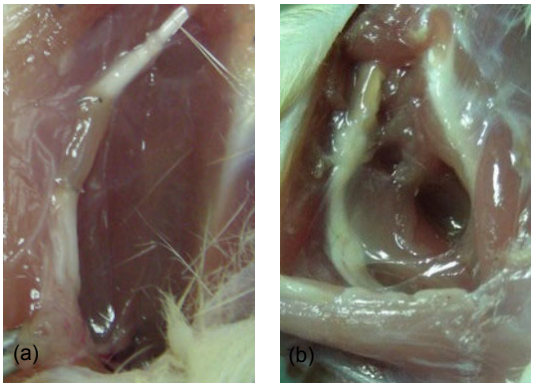

Fig. 2 Anastomosis with PHO tube and autograft conduits

(a) Day 60 PHO conduit; (b) Day 60 control group. There is marked fibrosis around the conduit in the control group compared to the PHO conduit

3.2 SFI analysis

SFI values were calculated from the data of the walking track analysis on Days 7, 21, 45, and 60 and median values were analyzed statistically (Table 1). In both groups there were significant falls in SFI percentages over time. On Day 45, the difference in SFI values between the control group and PHO group was found to be statistically significant. At the end of the implantation period, the control group had higher SFI

Table 1 Mean SFI values of groups on each day and their statistical analysis

Group	SFI (%)				Friedmann analysis
	Day 7	Day 21	Day 45	Day 60	
Control	−82.95±9.95	−72.71±9.39	−69.58±5.14	−47.91±6.97	p=0.005
PHO	−89.88±3.11	−78.87±13.61	−88.20±8.60	−61.96±14.07	p=0.026
Mann-Whitney U test	p=0.222	p=0.421	p=0.016	p=0.151	

values (close to zero) than the PHO conduit group, but the difference was not statistically significant.

3.3 Electrophysiological evaluation

For four electrophysiological parameters, experimental limb/control limb ratios were calculated on Days 21 and 60, postoperatively (Lago *et al*., 2007). There was a numerical increase in the amplitude of action potential and a decrease in duration period over time in the experimental group and control group. However, in both groups, the effect of day change or the group difference on the latency, CMAP amplitude, duration, or CMAP area was not found to be statistically significant.

3.4 Histological parameters

All regenerated axons in groups were analyzed histologically for two important factors: one is the neuroregeneration and the other is the soft tissue response of the PHO conduit and the inflammatory reaction around the autograft. Neuroregeneration was analyzed by nerve diameter measures, stained Schwann cell count, macrophage count within the nerve, and NFP staining within the nerve.

Schwann cell count in the PHO group and control group gradually increased over time (Days 7 to 60) and this difference was statistically significant within each group (Friedmann; p<0.01, p<0.05). For each individual day, there is a statistically significant difference between groups, showing the superiority of the autograft (p<0.001, F: 62.671) (Fig. 3).

When the staining pattern is concerned, on Day 60 there was homogenous Schwann cell alignment parallel to the regenerated axons in the PHO group (Fig. 4a). In the control group, the Schwann cell count is markedly high (Fig. 4b).

CD68 staining count was higher in the PHO group when compared to the control group for all days of implantation (Fig. 5). On Day 60, there was a decline in macrophage count for both groups. However, none of the results revealed statistical significance.

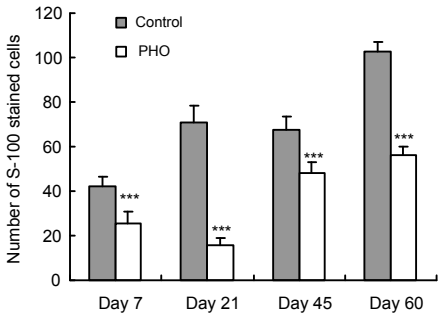

Fig. 3 Mean number of S-100 stained cells in the regenerated nerve in both control and PHO groups on designated days

For each individual day, there is a statistically significant difference between groups (*** p<0.001, F: 62.671)

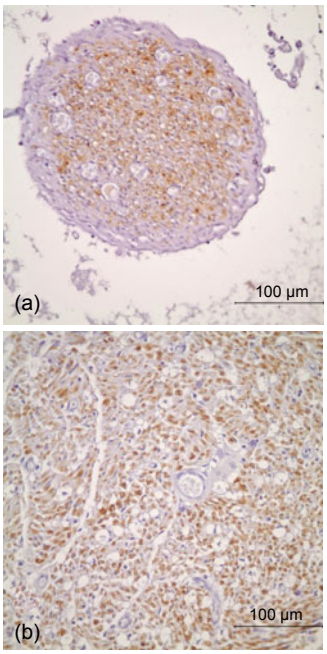

Fig. 4 S-100 staining of nerve graft in the PHO and control groups on Day 60, distal anastamosis side

(a) Day 60 PHO group: staining density increases and Schwann cell alignment is homogenous and parallel to the newly generating axons; (b) Control group: scattered Schwann cell staining

In the control group, on Day 7 the number of the NFP count was at a high value, and by Day 45 it

decreased to almost a similar value to the conduit group. In the PHO group on Days 45 and 60, the NFP count was found to be increased (30.00 and 42.67, respectively) and this increase was statistically significant (Friedmann; $p<0.05$). For each individual day, there is a statistically significant difference between groups from the aspect of NFP staining, indicating the superiority of the autograft ($p<0.01$, F: 21.973) (Fig. 6).

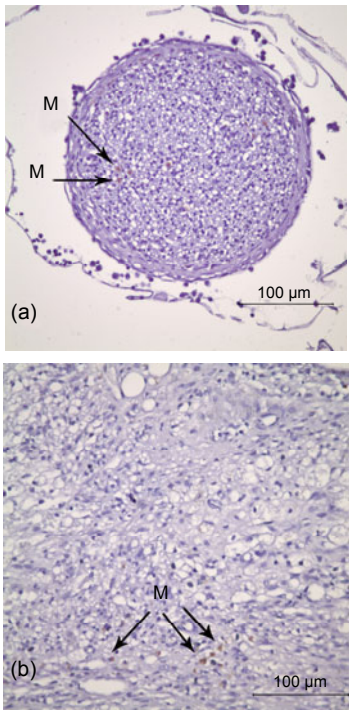

Fig. 5 CD68 staining of nerve graft in the PHO and control groups on Day 45, distal anastamosis side

(a) PHO regenerated nerve graft: macrophages are gathered in the center; (b) Control group: there are a few dispersed macrophages in the autograft. M: macrophage (arrows indicate dark brown-stained macrophage cells in both groups) (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

For nerve diameter measures, the control group had the highest values compared to the PHO group on each day (Friedmann; $p<0.05$) (Fig. 7), and this difference was found to be statistically significant.

On Day 21 of implantation, S-100, NFP and CD68 counts were found to be decreased, but on Day 45 there was a sharp increase for all parameters. A similar undulating pattern for implantation days was present in the PHO group for nerve diameter measures (Fig. 7).

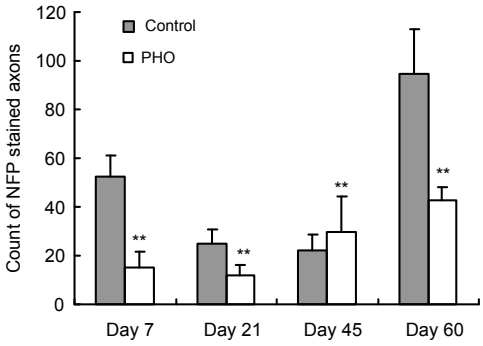

Fig. 6 NFP staining of regenerated axons (mean values) in the PHO and control groups on designated days

For each individual day, there is a statistically significant difference between groups (** $p<0.01$, F: 21.973)

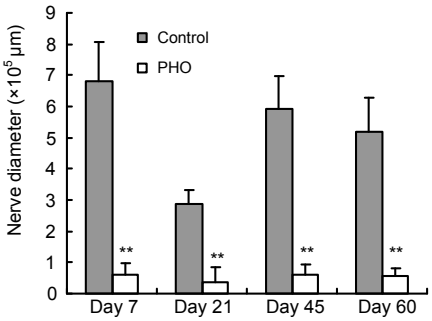

Fig. 7 Mean values of regenerated nerve diameter in the PHO and control groups

For each individual day, there is a statistically significant difference between groups (** $p<0.01$, F: 16.888)

Besides neuroregeneration parameters, we have also analyzed the soft tissue response of the PHO conduit and compared it with the inflammatory reaction formed around the autograft histologically. This inflammatory reaction was seen mainly as the capsule formation around the nerve segment. The surrounding capsule was analyzed for thickness, giant cell count and angiogenesis within the capsule of the conduit. In the PHO group, the surrounding capsule is thin for all days. Additionally, the giant cell count was prominent especially on Day 60 and was detected in the neighborhood of the PHO conduit (Fig. 8a). In the control group there was a thick capsule which was occupied mostly by polymorphonuclear cells on Day 7, and the number of inflammatory cells decreased by Day 60 (Fig. 8b).

When the two groups were compared, it was found that from Days 7 to 60, the capsule thickness was lower for PHO conduits than for the control

group and this difference was statistically significant. However, comparison of the two groups for capsule thickness on each day did not reveal statistical significance (Fig. 9).

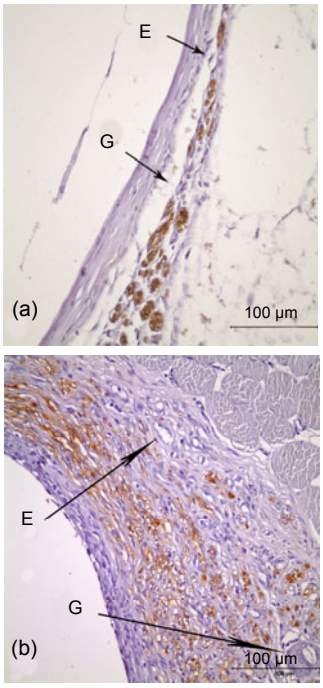

Fig. 8 Capsule surrounding the conduit in the PHO and control groups on Day 60 (CD68 staining, light microscope)

(a) Day 60 capsule section of PHO group: thin capsule with a loose supportive connective tissue behind; (b) Day 60 capsule section of control group: giant cells are increasing in amount and capsule is thicker and contains more inflammatory cells than the capsule of the PHO conduit. Arrows indicate the multinuclei giant cells (G) and endothelia cells (E), respectively

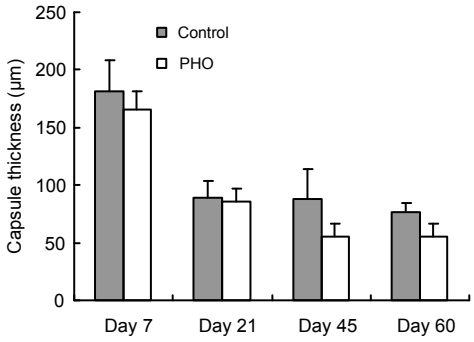

Fig. 9 Mean capsule thicknesses of autograft and PHO conduit on designated days

There was no statistical significance between groups on implantation days (p>0.05, F: 3.557)

For all immunohistochemical staining parameters (S-100, CD68, NFP, nerve diameter, capsule thickness), there was a significant difference between PHO and control groups independent of days except for capsule thickness (general linear models, p<0.05, p<0.01, p<0.001). Again for all parameters, groups vs. days interactions were found not be statistically significant.

3.5 In vivo degradation study of PHO conduits

Biodegradation of implanted PHO conduits was studied for molecular weight loss of the polymer conduit by gel permeation chromatography two months after implantation. It was found to be reduced from 257400 g/mol to 246600 g/mol after two months of implantation.

4 Discussion

In this present study, we have investigated the difference between autograft and PHO conduits in respect of soft tissue response and neuroregeneration. Neuroregeneration was analysed via SFI scores, electrophysiological data, and immunohistochemistry, and soft tissue response was evaluated via capsule thickness around the conduit.

We have conducted our research on the 1st, 3rd, 6th, and 8th weeks of implantation. It has been previously reported that nerve regeneration begins as early as Day 7 of anastamosis (Hazari *et al*., 1999; Chen *et al*., 2007) and therefore we have investigated the early regeneration of the nerve accordingly.

From the beginning of the 1st week to the 8th week, immunohistochemical and electrophysiological data presented a better neuroregeneration in the autograft group compared to the PHO conduit. This superiority in nerve regeneration might be due to several reasons. First of all, in the early period of implantation, the PHO group lacks Schwann cells, whereas nerve autografts have an inherent population of active Schwann cells. This condition is also present in the autograft for inherited axons, resulting in high NFP on Day 7 compared to the conduit group. Secondly, this high level of NFP count and nerve diameter measures gradually decrease to lower levels on Day 45, which might be due to shrinkage of the inherited degenerated axons in the autograft group.

This is a well known phenomenon in neuroregeneration, which is explained as the apoptosis of the newly regenerated axons that might innervate a wrong target. This finding is also supported by higher CMAP levels recorded on both Days 21 and 60 for the autograft, resulting in better motor functional recovery.

SFI scores decreased within time in both groups, meaning an improvement and superiority in neuronal function and regeneration as the implantation day reaches Day 60. Similarly, electrophysiological tests (CMAP, duration, and latency values) revealed neuroregeneration in both groups within the implantation days. For both functional recovery parameters, autograft was found to be superior compared to the PHO conduit group.

Although immunohistochemical and electrophysiological data presented a better neuroregeneration in the autograft group compared to the PHO conduit in our research, the PHO conduit has some superiority in several aspects. First of all, in the experimental group, the tube form of the PHO conduit forms a surface for contact guidance and keeps the neurotropic factors and microenvironment needed for neuroregeneration between the proximal and distal stumps of the dissected nerve. Schwann cells and macrophages produce neurotrophic factors, which support and guide the regenerating fibers (Mosahebi *et al*., 2002; Aguilar Salegio *et al*., 2010). These two cells play a pivotal role in two main landmarks of peripheral nerve regeneration: neurotropism and contact guidance (Mosahebi *et al*., 2002; Lewin-Kowalik *et al*., 2006). We observe a longitudinal Schwann cell and macrophage alignment along with regenerated axons parallel to the conduit. This finding was also supported with the motor functional recovery presented as a gradual increase in CMAP levels and a decrease in SFI values in the PHO group over time. It is possible that the regeneration rate in the PHO tubes increases as the Schwann cells from the proximal and distal stumps have re-established a line of cellular communication between the end organ and the cell bodies in the dorsal root ganglion.

In the study of Vleggeert-Lankamp (2007), a newly introduced biomaterial is primarily evaluated from the aspect of soft tissue response. The soft tissue response is detected mainly by the inflammatory reaction present around the material. We have also evaluated the soft tissue response of the newly formed PHO nerve conduit and compared it with the autograft. The capsule around the PHO graft is thicker on Day 7 with prominent inflammatory cellular diversion and neovascularization. This acute intense inflammatory response subsided in two months. On Day 60 of implantation, the capsule thickness seemed to be lower than that of the autograft. However, this difference was not statistically significant. This data points out that the PHO conduit reveals good soft tissue response.

The biodegradation process of biomaterials following in vivo application was explained by the transfer of water molecules from extracellular space into the polymer structure (Stock *et al*., 2000; Lenz and Marchessault, 2005; Lu *et al*., 2007; Liu and Chen, 2008; Orts *et al.*, 2008). This process of biodegradation for the PHO graft was found to be quite long (Marois and Zhang, 1999; Hazer *et al*., 2012b). We have also achieved similar results to these literatures: a negligible molecular weight drop of 10000 g/mol after two months of implantation was detected. These types of conduits should at least stay for three months until the regeneration is almost completed without degeneration (di Summa *et al.*, 2011). However, a longer presence of the conduit around the regenerated nerve might decrease the free movement of the nerve within the muscle and therefore increase the malfunction of the regenerated nerve (Dahlin *et al.*, 2001). This is also a benefit for nerve grafting in the chronic phase of regeneration, in order to maintain the microenvironment of nerve regeneration and maintain the contact guidance and the alignment of the regenerating axons.

Overall, immunohistochemical and electrophysiological data presented a better neuroregeneration in the autograft group on Day 60 of implantation. In the PHO conduit group, there was neuroregeneration at the end of Day 60, but the degree of regeneration was not found to be as good as in the autograft group. As a result, although SFI data might be promising, PHO conduits probably do not have much potential as a nerve conduit compared to current conduits presented in the literature (Lewin-Kowalik *et al*., 2006; Vleggeert-Lankamp, 2007).

5 Conclusions

To avoid the disadvantages of autograft such as donor-site injury, tissue engineering conduits of

different materials were developed to reconstruct the injured nerves. In this study we have investigated a microbial polyester, PHO, as artificial nerve conduits for up to 60 d. PHO nerve conduits revealed good soft tissue response. However, immunohistochemical and electrophysiological studies showed less neuroregeneration compared to autograft. Further studies can be conducted with a modified (cell culture or growth factor embedded forms) PHO conduit for a longer duration of implantation to improve neuroregeneration.

Compliance with ethics guidelines

D. Burcu HAZER, Ercan BAL, Gülay NURLU, Kemal BENLI, Serdar BALCI, Feral ÖZTÜRK, and Baki HAZER declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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