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Developing a Model of Peripheral Nerve Graft Based on Natural Polymers

Abstract
Presented are the results of investigations into the preparation of a peripheral nerve prosthesis. The prosthesis is built up of a multichannel core having a diameter of 2 to 5 mm. The core prepared by freeze drying is housed in a polymeric sleeve. The prosthesis core is made of microcrystalline chitosan (MCCh) while the sleeve is prepared from poly(DL-lactide-co-glycolide) copolymer. The usefulness of the prepared biomaterial was assessed by in vivo testing on animals

Key words: *biopolymers, peripheral nerve, prosthesis, chitosan.*

Introduction

Peripheral nerves are often seriously impaired or even disrupted in the ever increasing number of injuries, occurring mainly in traffic accidents. It may significantly affect the motoric system and cause atrophy of the denervated muscles. Unprotected fibres in the proximal segment of the disrupted nerve have the ability to regenerate. The process is, however, spontaneous and chaotic, leading in most cases to neuroma or microneuroma [1, 2]. Disordered sensory fibres build improper joints causing mechanical and chemical excitations resulting in pain sensation [6, 7]. The phenomenon is known as neuropathic pain resistant to pharmacological therapy and poses a serious therapeutic problem [7].

The problem of repairing extensively impaired peripheral nerves is being studied in many research centres throughout the world. The most difficult clinical problems are the so-called "gap injuries" in which the gap in the nerve continuity hinders its immediate sewing [1]. The gaps are often bridged with a nerve fragment taken up primarily from the patient's skin with the negative effect of additional mutilation. Construction of nerve prostheses, or more precisely, of nerve graft substitutes, would be the optimal solution to the problem. An ideal prosthesis is expected to satisfy following demands: 1. display neutral immunity, 2. allow easy joining with the nerves stumps, 3. be elastic, 4. have an internal multichannel structure enabling the re-growth of the parallel nerve fibres, 5. be tight enough to prevent the in-growth of connective tissue, 6. enable the incorporation of neuralgic and neurotropic substances to the prosthesis structure, and 7. indicate slow biodegradation and resorption of the material [1 - 6]. Up to now, so-called natural tunnels have been mostly used in nerve repair with varying success, most notably:

- connective tissue/collagen, hollow or filled with fibrin,
- synthetic: silicone or polyethylene with varied permeability of the walls [1, 5, 6,10, 11].

Attempts were made to use tunnels with parallel arranged carbon fibres or fibres made up of resorbable poly(glycolic acid) to improve the orientation of the regenerating nerve fibres [1, 7]. These tunnels were also filled with neurotropic substances and/or populations of various cells (Schwann cells, mother cells) that are subjected to various modifications. In the last two years, tubes and nerve prostheses without an external coat (substitute of perneurium) prepared from sole chitosan or alginate have also been investigated [2 - 4, 8, 9, 10, 12, 13]. A hydroxyapatite-chitosan complex was used and the cross-section of the prosthesis changed to a triangle to improve the tenacity of the tubes [14, 15]. Tests conducted on animals have shown that the chitosan tubes induce an effective regeneration of the peripheral nerves and undergo a stepwise resorption without inflammatory reactions [10]. In the therapy of spinal cord damage in animals, interesting investigations on the preparation of an implant of poly–β-hydroxy butyrate with a content of alginate hydroxy-gel enriched with fibronectin are presented [16, 17]. A lyophilised alginate gel covered with a mesh made of poly(glycolic acid) was used in the regeneration of extensively damaged nerves in cats. That solution allowed the perfect regeneration of the damaged nerves; however, strong inflammatory reactions could be observed during the process of implant biodegradation [2]. In other, more inspiring experiments on animals, fragments (few millimetres) of the spinal cord were removed and the resulting gap was instantly bridged with an alginate sponge [14].

Nowadays, only one commercial artificial nerve is used in human therapy under the trade name of NeuraGen^{R}. The implant is made up of type I collagen in the form of a hollow tube and is only suitable for bridging short (3 - 5 mm) nerve defects [1, 5]. This explains why many research centres are working on the preparation of more efficient solutions to the problem.

The aim of the research presented was to elaborate a model of nerve prosthesis that showed neutral immunity, was biodegradable and resorbable, and with an internal structure allowing facilitated regrowth of the parallel nerve fibres. The prosthesis is expected to prevent the patient from further suffering caused by the uptake of a nerve fragment from his body for the reconstruction. The authors hope to identify the preparation of a prototype of a multichannel prosthesis based on natural polymers; chitosan from the poly-amino-saccharide family in particular. Such construction of the implant and the used material were adopted on the basis of a literature search and an introductory investigation by the authors, which had highlighted the beneficial impact of the microcrystalline form of chitosan upon the regeneration of nerve fibres [18].

Materials

Microcrystalline chitosan (MCCh)

Microcrystalline chitosan was prepared according to a method elaborated at IBWCh [19]. MCCh of high purity, with varying molecular mass and pH, was used in the investigation.

The physicochemical parameters of the prepared MCCh are as follows:

- MCCh/278: M_v = 423 kD, deacetylation degree (DD) = 82%, polymer content = 2.5%, water retention value (WRV) = 4600%, ash content = 0.09%, pH = 7.0,
- MCCh/171: M_v=287 kD, DD = 80%, polymer content = 2.5%, WRV = 3990%, ash content = 0.04%, pH = 7.5.

Resomer RG 755S - poly(DL-lactide-co-glycolide) copolymer

Copolymers of D,L-lactide and glycolide type Resomer RG 755S supplied by Boehringer Ingelheim Co. were used in the research. They were characterised as having the following composition: 75 mol% of DL-lactide and 25 mol% of glycolide residues. Inherent viscosity = 0.68 dl/g.

Polypropylene fibres

The PP fibres were melt-spun on an extruder spinning bank, which is in the possession of IBWCh. The prepared monofilament PP fibres exhibited a good smoothness and their set diameter was between 0.16 and 0.22 mm. The fibres served as temporary component of the matrix and were removed after the channel in the prosthesis core had been formed. Therefore, the mechanical parameters of the fibres were not measured.

Silicon mould

Commercial silicone sleeves with a diameter between 2 and 5 mm were used in the construction of the matrix of the prosthesis core.

Glycerol

Glycerol (Fluka Co.), analytically pure according to Ph. Eur., was used as chitosan plasticiser.

Methods

Estimation of the average molecular mass of chitosan (M_v) - Viscometric method

The viscometric average molecular mass was calculated on the basis of intrinsic viscosity number [η] values. Viscosity was measured on a dilution viscometer with capillary No. 1, K ≈ 0.01 at 25 ± 0.1 °C. The average molecular mass was calculated using the Mark-Houwink [20] equation.

Estimation of the deacetylation degree of the chitosan (DD) - method of the first derivative of UV spectrum analysis

The deacetylation degree was estimated through a spectrophotometric method in which the maximum of the first derivative was found of the UV spectrum, which led to the calculation of DD according to a procedure prepared at IBWCh.

Estimation of ash content

Ash content in chitosan was estimated gravimetrically after annealing the polymer at 800 °C according to the IBWCh procedure.

Estimation of water retention value (WRV)

WRV was estimated according to standard method [21].

Estimation of polymer content in the microcrystalline chitosan (MCCh)

The polymer content in the MCCh was estimated gravimetrically according to the IBWCh procedure.

Estimation of heavy metal content

Heavy metal content was estimated with the use of Atomic Absorption Spectroscopy (AAS).

Assessment of mechanical properties

Tests of mechanical properties were conducted in the accredited Laboratory of Metrology of IBWCh – PCA certificate No AB 388 Metrological Testing.

- nerve prostheses made by freeze drying were tested according to ISO standard 7198:1998
- prosthesis sleeves made up of poly(DL-lactido-co-glycolide) copolymer film were tested according to standards PN-ISO 4593:1999 (film thickness), PN-EN ISO 527-3:1998 (strength parameters).

Inspection of the structure

The surface and cross-section of the nerve prostheses were analysed by means of Scanning Electron Microscope SEM Quanta 200 (FEI Co., USA).

In vivo testing on animals

Biological *in vivo* examinations were performed in the specialised medical unit - Department of Physiology of the Silesian Medical University, Katowice.

The functional assessment of rats' peripheral nerve regeneration with prostheses was conducted in the region of extensive defects in the sciatic nerve. The experiments were carried out with Wistar C male rats.

In all anaesthetised animals (Avertine) the right sciatic nerve was exposed and its fragment, 7 mm in length, was removed. The defect was then replaced with a prosthesis fragment of a similar length, which was affixed to the perineurium of the proximal and distal stump (***Figure 1***). The prosthesis was filled up with saline, and then the postoperative wound was sutured.

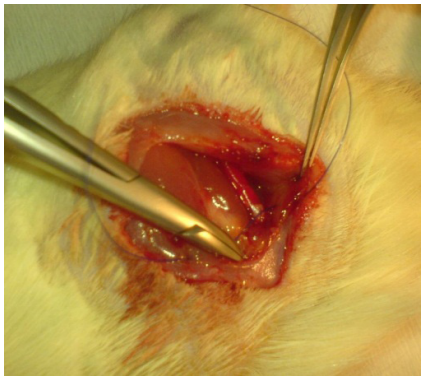


Figure 1. *View of the in situ grafted multichannel chitosan prosthesis.*

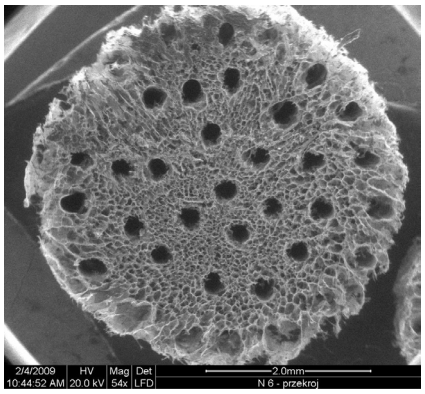
The animals were euthanised after seven weeks and the grafted prosthesis was collected, together with the adjoining fragments of the proximal and distal sciatic nerve.

Preparing the specimens for histological assessment

Nerve fragments were fixed together with the prostheses in a formalin solution, dewatered in a saccharose solution and immersed in a medium for the histological experiments, before being frozen. The prepared specimens were cut transversally or longitudinally with a freezing microtome into tiny scraps, 10 μm thick, and placed onto microscope glass plates.

Histological assessment of prosthesis – grafted nerves

The histological assessment of prosthesis-grafted nerves was performed using light and fluorescence microscopy.



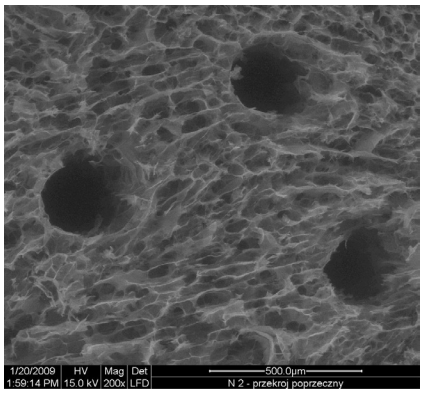


Figure 2. *SEM microphotographs of the core cross-section of the multichannel prosthesis of peripheral nerve made of MCCh by means of freeze drying. Diameter of the prosthesis – 5 mm; diameter of the channels – 0.25 mm.*

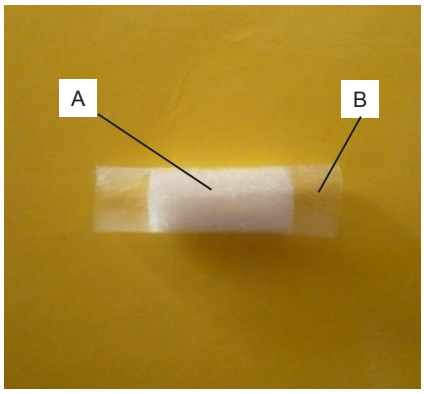


Figure 3. *Prosthesis of a peripheral nerve prepared by freeze drying; A-multichannel core, B- outer sleeve.*

Assessment of the extent of neuropathic pain

Assessment of the extent of neuropathic pain was done on the basis of the autotomy phenomenon occurring in animals (gnawing the denervated paws' toes due to the neuropathic pain).

Results of investigation

Preparation of a peripheral prosthesis model

The prosthesis core was made of microcrystalline chitosan by means of a freeze drying technique [22]. An assumed number of smooth tensioned polypropylene fibres was placed in the interior of a silicon mould, 2 or 5 mm in diameter. The mould was then filled up with a suspension of microcrystalline chitosan and frozen at –25 °C for 15 minutes. Next, the silicon mould was removed and the obtained cylindrical core with the fibres was subjected to freeze drying. PP fibres were removed after drying leaving a prosthesis, 5 - 10 mm long with a diameter of 2 or 5 mm, containing channels in an amount depending upon the number of the earlier inserted (and removed) fibres. After grafting, the parallel channels serve to indicate the growth direction of the disrupted nerve fibres (***Figure 2***). To ease the connection of the prosthesis with the stumps of the defected nerve, the formed multichannel core was inserted in a sleeve made up of a resorbable polymer. The sleeve reached beyond the core for 4 mm on each side (***Figure 3***).

The prosthesis sleeves were made of a film of either microcrystalline chitosan (MCCh) or Resomer RG 755S poly(DL-lactide-co-polyglycolide) copolymer. MCCh containing 1.7% of the polymer and with pH of 6.1 - 6.2 with the addition of glycerol was used to prepare the sleeves for the nerve prostheses. The gel was cast onto Teflon plates and dried for 24 hours at ambient temperature. The obtained film was neutralized with Na_2CO_3 solution containing glycerol, and rinsed with a mixture of water and glycerol, before being freeze dried for 24 hours at -25 °C and under pressure of 0.1 - 0.57 hPa.

For the preparation of a film of poly(DL-lactide-co-polyglycolide), solutions of the copolymer in 1,4-dioxan with the addition of polyethylene glycol 600 were used. The solutions were cast on Teflon plates, dried for 24 hours at ambient temperature and then for 4 days at 50 °C, under a pressure of 0.07 MPa, until the complete removal of the solvent.

It was found during the investigation that the proper selection of the diameter of the PP fibres that shape the channels of the prosthesis is of profound importance. Fibres with a diameter of ca. 0.16 mm formed channels with too small a diameter, which were prone to deformation and closure after the freeze drying. The use of 0.22 mm diameter PP fibres produced better results allowing the formation of prostheses with evenly arranged channels with uniform diameters (***Figure 2***).

Silicone moulds with 2 and 5 mm diameters were used in the preparation of the prostheses. With the 5 mm moulds, cores could be made with as many as 37 channels. However, as a result of consultations with neurosurgeons, it was decided to fix the prosthesis with a diameter of 2 mm. With such a small diameter, prototypes of the implant were prepared containing from 7 to 13 channels.

It appeared that, during the formation of the prosthesis, a proper content of the polymer in the microcrystalline chitosan is important as well. The use of MCCh containing 2% polymer yielded prosthesis with a developed surface, while a 2.5% concentration of the polymer resulted in a more compact structure. Such a structure is expected to largely prevent the overgrowth of connective tissue through the prosthesis surface.

Estimation of the mechanical properties of peripheral nerve prostheses

Mechanical properties of the core of the peripheral nerve prosthesis made of MCCh by freeze drying were examined (***Table 1***). The biomaterial of MCCh/278

Table 1. *Mechanical properties of the core of chitosan prostheses with 7 and 13 channels.*

Tested parameter	Test results	
	7 channels	13 channels
Outer diameter, mm	1.72	1.76
Thickness variation coefficient, %	6.05	6.48
Max. drawing force, N	4.42	4.02
Drawing force variation coefficient, %	28.5	31.4
Tenacity, MPa	6.21	5.65
Elongation at max. stress, %	0.622	0.708
Elongation variation coefficient, %	49.6	55.5

Table 2. Mechanical properties of film made of MCCh and poly(DL-lactide-co-glycolide) copolymer.

Condition	Parameter tested	MCCh	poly(DL-lactide-co-glycolide) copolymer
Dry	Film thickness, mm	0.83	0.052
	Thickness variation coefficient, %	13.3	14.0
	Max. drawing force, N	58.9	59.8
	Drawing force variation coefficient, %	21.7	19.9
	Tenacity, MPa	47.2	69.0
	Elongation at max. stress, %	43.0	49.3
	Elongation variation coefficient, %	27.9	21.9
Wet (immersed in demi-water for 60 s)	Film thickness, mm	0.84	0.058
	Variation coefficient of thickness, %	2.38	14.7
	Max. drawing force, N	8.90	10.6
	Drawing force variation coefficient, %	26.1	32.3
	Tenacity, MPa	7.04	13.8
	Elongation at max. stress, %	114	94.6
	Elongation variation coefficient, %	17.9	12.8

was in the form of a cylinder 7 mm long with ca. 2 mm diameter, with 7 or 13 channels. Tests revealed that the core of the prepared prosthesis shows a tenacity above 5 MPa (it was somewhat better in the 7-channel version). Such tenacity ensures safe bridging of the defected nerve. When dry, the prostheses revealed a rather low elasticity, which is expected to improve after being implanted, filled with saline and contacted with systemic fluids. It should, under such conditions, not impair the limb's mobility.

The sleeves of the nerve prostheses in the form of a film made up of microcrystalline chitosan and Resomer RG 755S were also tested. The goal of the testing was to fix variation of mechanical resistance parameters of the film in dependence on the environment. It was especially important to learn whether the mechanical resistance of the prostheses materials will remain on the level acceptable for a correct joining with the perineurium after the grafting to a living organism (moist environment). Results of the testing are shown in ***Table 2***.

Results presented in ***Table 2*** show a substantial decrease of tenacity of both films in moist conditions. The drop amounts to 85% and 80% for the MCCh film and Resomer RG 755S films, respectively. On the other hand, elasticity was improved in wet conditions by 71% and 45% for the MCCh and Resomer films, respectively. It can also be seen that the films of Resomer are about 49% stronger than those of chitosan in wet conditions. This was the reason that the films of Resomer RG 755S were selected for the construction of the outer sleeve in the further experiments.

Biological assessment of the multichannel prostheses of peripheral nerves made of microcrystalline chitosan

The biological examination of peripheral nerve prostheses was carried out on Wistar C rats, of which three experimental groups were formed:

- **Group 7A** - 11 animals with implanted chitosan prosthesis 7-channels. Implant core - MCCh (Mv = 423 kD, pH = 7.0), Implant sleeve - Resomer RG 755 S.
- **Group 7B** - 8 animals with implanted chitosan prosthesis 7-channels. Implant core - MCCh (Mv = 287 kD, pH = 7.5), Implant sleeve - Resomer RG 755 S.
- **Group 13A** – 9 animals with implanted chitosan prosthesis 13-channels. Implant core-MCCh (Mv = 423 kD, pH = 7.0), Implant skin - Resomer RG 755 S.

In the identification symbol, the number identifies the amount of channels and the letter describes the kind of MCCh used.

Examination of the medical suitability of the peripheral nerve prostheses confirmed that the prototypes chosen for the experiments did not pose any difficulty in the course of grafting. The sleeve of the prosthesis provided the chance of easy and proper joining of the implant with the perineurium. The multichannel chitosan core revealed perfect air permeability.

Autotomy assessment of the animals after the implantation was made, which serves as an indication of the neuropathic pain. The extent of autotomy was assessed using the 13-point Wall scale. The examination did not reveal any essential difference in the extent of autotomy between individual groups of the animals (***Figure 4***). The average extent of autotomy in the three groups of animals after the implantations of selected prosthesis prototypes was in the range of 1.89 to 3.00 in the 13-point Wall scale. Hence, it may be deduced that the sensory fibres

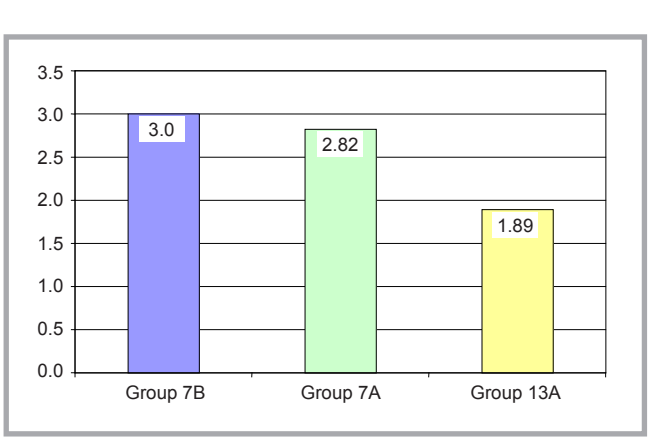


Figure 4. *Intensification of neuropatic pain of animals.*

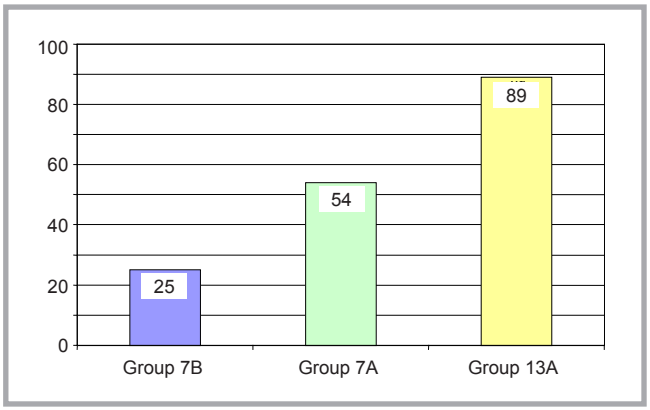


Figure 5. *The percentage of animals in which nerve growth into the prosthesis was observed.*

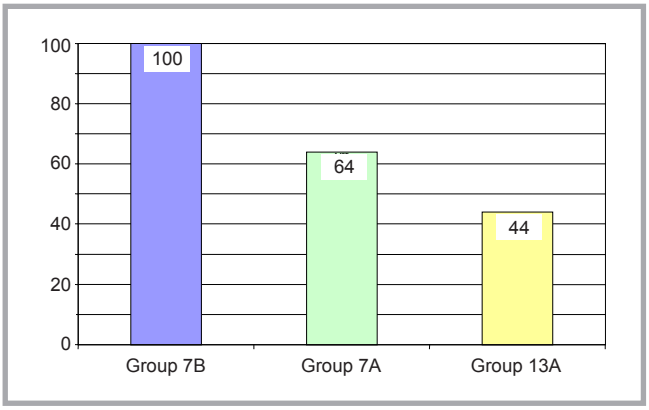


Figure 6. *Percentage of animals with a granulocytic infiltration in prosthesis core.*

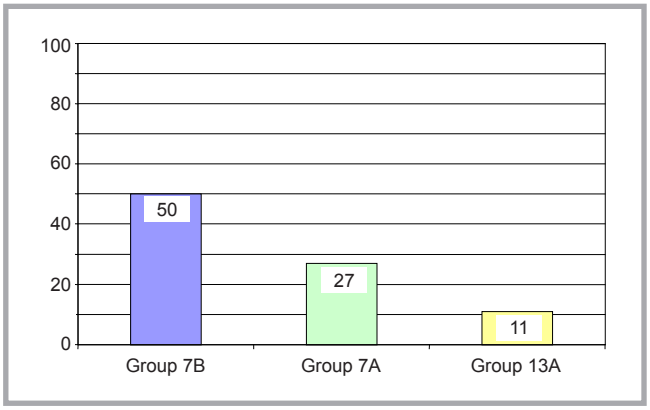


Figure 7. *Percentage of animals with the utmost inflammation (extent of 5 or 6).*

of the prosthesis-generated nerves do not cause strong mechanical and chemical excitations which evoke pain. It must be stressed that subsequent morphological assessment concerning regeneration of the peripheral nerve showed that the intensification of autotomy in the test groups of animals corresponded with the regeneration degree of the nerve. The better the organisation of the regenerating nerve fragment the lower the autotomy, meaning a milder neuropathic pain.

Histological examination

Assessment of the growth of nerve fibres into the prosthesis channels

The percentage of animals in which the nerve grew from its edge on a length of at least 2 mm into the depth of the prosthesis counting from the end of the proximal nerve was estimated. It was found that the highest growth of the nerve into the prosthesis reached the highest percentage of about 89% of animals in group 13A including 9 rats. These animals were grafted with 13-channel prostheses made of microcrystalline chitosan marked MCCh/278. The lowest growth of the nerve fibres occurred in group 7B. In that group, the percentage of rats in which the growth of the nerve reached 2mm into the prosthesis was as low as 25%. The animals in the 7B group were grafted with a 7-channel prosthesis made of microcrystalline chitosan marked MCCh/171. In group 7A, in which the animals were grafted also with the 7-channel prostheses but made of the microcrystalline chitosan MCCh/278, the percentage amounted to 54%. Since the chemical purity of both chitosans MCCH/278 and MCCh/171 is equal, it may be assumed that, with the same number of channels, the rate of nerve growth depended upon the pH of the material. This was 7.5 and 7.0 for MCCh/171 and MCCh/278, respectively. Supposedly, the parameter plays an important role in the process of nerve regeneration. However, the presumption calls for proof in further research.

Assessment of inflammatory reactions of the prosthesis

The second step of the histological assessment was concerned with the examination of the character (granulocytic or lymphocytic infiltration) and extent of inflammatory reactions in the prosthesis core. It is known from a literature review that the lymphocytic inflammatory response is beneficial to the regeneration of nerves thanks to the delivery of cytokines by the activated lymphocytes influencing the axonal re-growth [23]. On the other hand, an infiltration composed of granulocytes exerts a negative impact upon the regeneration of nerves, ultimately leading toward destruction (lysis) of the prosthesis material.

A semi-quantitative assessment was applied (scores from 0 to +++) separately for each type of prosthesis, and presented as a sum of the +'s in a scale range from 0 to 6. Results are shown in ***Figures 6*** and ***7.***

It was found that the granulocytic infiltration emerged in the prosthesis core only. The highest percentage of animals with granulocytic infiltration (100%) was observed in group 7B grafted with the 7-channel prosthesis made of MCCh/171. In the same group of animals, the highest total inflammatory state (granulocytic + lymphocytic) was found. The extent of total inflammation of 5 or 6 (in the scale 1 - 6) occurred in 50% of the animals (***Figure 7***). In the case of prostheses with the same number of channels but made of MCCh278, the number of animals (group7A) with granulocytic infiltration was 36% lower, and the percentage of those with the utmost inflammation was nearly 50% lower.

The results confirm the assumption according to which the environment's pH plays an important role in the regular regeneration of nerves. The lowest percentage of animals in which the inflammatory state occurred after grafting was recorded in the case of the 13-channel prosthesis. In the animal group grafted with that prosthesis (13A), the granulocytic inflammatory condition was found in 44% and the total one (granulocytic + lymphocytic) with prevailing lymphocytic in 11% of the animals.

Assessment of the resorption of prostheses implanted to animals

The third stage of histological examination covered an assessment of the biodegradation process proceeding in the animal organisms. The resorption process was traced by assessment of the infiltration of macrophages, which plays a paramount role in the regeneration of nerves. A semi-quantitative assessment was performed of the proliferation of the connective tissue in the prosthesis interior (scores 0 to +++). The results of the examination reflect the summarised results in a given group related to the maximal (3× number of animals in the group) possible result. The results are presented in ***Figure 8.***

On the basis of the examination results presented in ***Figure 8***, no substantial differences were found between the particular animal groups in terms of the intensification of resorption processes of the nerve prostheses. In all groups of animals grafted with chitosan prostheses, the percentage of those in which the resorption

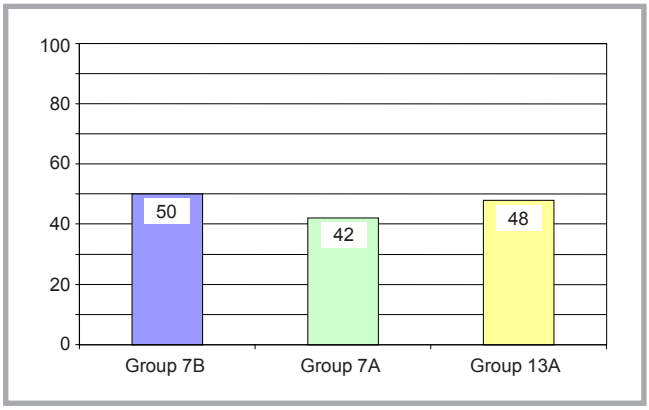


Figure 8. *Intensification of biodegradable implants.*

had been enhanced, was contained in the range from ca. 42% to 50%.

Conclusions

1. The positive results of the investigation confirm that the prepared prosthesis of the peripheral nerve is a promising candidate for use in neurosurgery.
2. It is advisable to continue the research with the goal of a further improvement of the implant, optimisation of the material parameters and estimation of the medical usefulness of the prosthesis throughout the entire regeneration cycle.

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