

Original article ■

# Analysis of Fascicular Structure and Connective Tissue Sheaths in Sural Nerve during Aging

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## SUMMARY

The aim of our study was to analyze the changes of connective tissue sheaths of epi-, peri- and endoneurium of sural nerve during aging.

The study was conducted on sural nerve samples of 10 cases aged 9-80 years. The specimens were embedded in paraffin using standard procedures, after which 5- $\mu$ m-thick cross-sections of nerve trunks were made and stained using Masson's trichrome staining. After morphological analysis of fascicular structure and connective sheaths of the nerve, morphometric analysis was conducted using the software for digital image analysis "ImageJ". Each investigated case was analyzed for total neural, epineurial and fascicular cross-section area, mean values of perineurial index, volume density of myelinated axons and of endoneurial content. To test the difference in mean values for statistical significance we used the Student's T-test for small independent sample.

The number of fascicles was 5-13, while the majority of the nerves had less than 10 fascicles. Fascicular structure, which included the number of fascicles and epifascicular/fascicular area ratio, did not show significant changes during aging. Perineurial thickness/fascicle size ratio statistically significantly increased in the older investigated group ( $p < 0.05$ ). Myelinated fibres were of smaller diameter, with more irregular form and markedly less frequent in older cases. Quantitative analysis showed statistically significant decrease in volume density of myelinated fibres in the older group.

As results of applied investigation methods we found thickening of perineurial sheath of sural nerve during aging, as well as endoneurial fibrosis. Future investigations of age-related changes should focus on analysis of the components of extracellular matrix within perineurium and endoneurium.

**Key words:** sural nerve, aging, degeneration, perineurium, endoneurial fluid

## INTRODUCTION

Spinal and cranial nerves are complex structures composed of myelinated and unmyelinated nerve fibres, grouped together in fasciculi, which connect central nervous system with peripheral tissues using electrical signals. Nerve fibres are surrounded by three connective tissue sheaths, the epi-, peri- and endoneurium which are continuous with the dura, arachnoid and pia mater. The outer layer is the epineurium, which is composed of the dense irregular connective tissue, ample of collagen fibrils and blood vessels. Perineurial sheath is specific fibrocellular layer which is made of a various number of perineurial lamellae (up to 20 in mammals), which are divided by narrow intercellular clefts containing collagen and elastic fibrils (1). Each perineurial lamella is bounded by basal lamina on both sides. The thickness of the perineurium ranges from 1,3 to 100  $\mu\text{m}$  (2). Endoneurium is the innermost layer which covers a single nerve fibre with its axon and covering Schwann cell that produces collagen fibrils. It also contains fibroblasts, mast cells and capillaries (3). The endoneurial fluid pressure is elevated inside the fascicle which minimizes endoneurial contamination by harmful substances from the surrounding tissue.

Aging shows a great impact on deteriorating morphologic and functional characteristics of peripheral nervous system. Functional age-dependent deficits may consequently appear as the features of structural and biochemical disorders of nerve fibers (degeneration of nerve fibers) that cannot be improved due to lowered capacity of reparation and regeneration mechanisms (4). Beside axons, extracellular matrix is also changed by aging process and, as it is involved in cell proliferation, migration and shape formation, it plays an important role in maintaining the functionality of peripheral nervous system.

In this study, therefore, we investigated the age-related structural changes of connective tissue layers of human sural nerve using the morphometric techniques.

## MATERIAL AND METHODS

For investigation we harvested a part of sural nerves' trunks of 10 cases aged 9 to 80 years, of both sexes, routinely dissected at the Institute of Forensic Medicine in Niš. The studied cases were not diagnosed as having neurological disorders nor diabetes during lifetime. The samples of sural nerve were taken in each subject in the following way: 5 cm long skin cut was made between the lateral malleolus and calcaneal tendon; after removal of the outer skin layer and subcutaneous tissues 3 cm long part of sural nerve trunk was harvested and afterwards fixed in 10% neutral-buffered formalin for 24 hours within one hour after removal. The specimens were embedded in paraffin using standard procedures. Serial transverse sections were 5  $\mu\text{m}$  thick and

cut on a microtome with a disposable blade for Masson's trichrome staining.

Morphologic analysis of all sections was done by microscope "Olympus C011" after checking for artefacts and pathologic appearance under light microscopy. We investigated the epineurial, perineurial sheath and endoneurial content. Afterwards, the images were made using x40, x100, x630 and x1000 objective on 5-megapixel colour digital microscopy camera. The images were used for morphometric analysis by Image J image analyzing software (<http://rsb.info.nih.gov/ij/>). Prior to every measurement spatial calibration was done using the object micrometer (1:100) for every magnification. Each investigated case was analyzed for the total neural, epineurial and fascicular cross-section area, mean values of perineurial index, volume density of myelinated axons and volume density of endoneurial content. At the smallest magnification, the profile of whole cross section of the nerve was circled using the option "polygon selections" from the main menu of the program (which is used for measuring the value of the total cross section neural area), and afterwards the same procedure was repeated for each particular fascicle (after which the total cross section fascicular area was measured). The values of total cross section epineurial area were obtained by subtracting the total cross section fascicular area from the total cross section neural area for each investigated case. Taking into the consideration that there are literature data (5, 6) about the influence of fascicle's size on perineurial thickness (greater fascicles have thicker perineurial sheath and vice versa), we decided to calculate the perineurial index (P index). The image of each fascicle in section of investigated nerve was taken at magnification of 100x. Using "straight line" selection outer diameter (the distance between two spots on the opposite sides of outer perineurial surface which passes through the center of fascicle- $D_o$ ) and inner diameter (the distance between the opposite two spots on the inner perineurial surface which passes through the center of the fascicle- $D_i$ ) of each fascicle was measured, at three different places. After three measurements, P index for each fascicle was calculated using the formula:  $P \text{ index} = [(D_o - D_i)/D_o] \times 100$  (6). We calculated P index for each investigated case from the obtained values of P index for each particular fascicle. In the third part of our study we investigated the ratio of myelinated fibres and endoneurial content by calculating their volume density. For each studied case we took the images of 10 fields of view inside different fascicles (magnification 1000x) by the random choice method. Analysis was performed using the plugin Grid Overlay (<http://rsbweb.nih.gov/ij/plugins/graphic-overlay.html>) whereby the test system grid was placed over the image. Characteristics of the test system are: total number of test points  $Pt=972$ , test system area  $At=8748 \mu\text{m}^2$  and distance between two neighboring test points  $d=3 \mu\text{m}$ . The points falling either on myelinated fibres (axon + myelin sheath) or endoneurial content were counted using cell counter plu-

gin. Volume density was calculated by using the formula  $V_{vf} = P_f/P_t$  where  $V_{vf}$  is volume density of investigated phase,  $P_f$  is the number of points falling on investigated phase and  $P_t$  is the total number of test points (7).

For the purpose of investigating the influence of aging on examined morphometric parameters, we divided all the cases into two groups (five cases each) which means that age was statistically different between the groups (group 1  $37 \pm 17$  years, group 2  $72 \pm 5$  years). To test the difference in mean values for statistical significance, we used the Student's T-test for small independent samples. This was done using SPSS for Windows version 20.

## RESULTS

Results of the morphometric analysis of all investigated cases are shown in Table 1. Sural nerve belongs to the group of oligofascicular nerves according to the number of fascicles (up to 10), with the exception of two cases (36 and 72 years old) which had 13 fascicles in the cross section. The mean number of fascicles between examined groups did not differ statistically (Table 2). Epi- and interfascicular part of epineurial sheath was clearly observable at histological samples, containing predominantly connective tissue and blood vessels (Figure 1). The fascicles were mainly round and oval in section and predominantly middle-sized (mesofascicles) (Figure 1). Morphometric analysis of the total cross section neural area, especially epifascicular and fascicular area did

not show statistically significant differences between the groups (Table 2), though the greater presence of adipocytes was observed in the epineurial layer of older group cases (Figure 1).

Perineurial sheath consists of several lamellae of thin perineurial cells, with rare nuclei of fibroblasts and blood vessels, which distinctly separate endoneurial content from the surrounding connective tissue (Figure 1). In younger group cases it was noticed that the thickness of perineurial layer was related to the size of the fascicles (the greater fascicles had the thicker layer), while in older group we detected fascicles with smaller diameter who had disproportionately thick perineurial sheath (Figure 2a, b). The analysis of mean values of perineurial index in both group cases showed statistically higher values in group 2 ( $p < 0.05$ ) (Table 2).

Observing endoneurial compartment of the sural nerve fascicles, the presence of various sized myelinated fibres was noticed, as well as connective tissue with Schwann cells and fibroblasts nuclei and the blood vessels localized mostly subperineurially or within intrafascicular septa. In younger cases myelinated fibres were bigger, round and more dense (Figure 2c), while connective tissue were more compact and scarce. Older group showed less frequent myelinated fibres (Figure 2d). Detected morphological varieties in appearance and density of myelinated fibres were confirmed by statistically significant difference in their volume density. Mean volume density of myelinated fibres in group 1 was  $33.54\% \pm 12.34$ , while it was significantly lower ( $p < 0,05$ ) in group 2 –  $14.31\% \pm 8.61$  (Table 2, Figure 3).

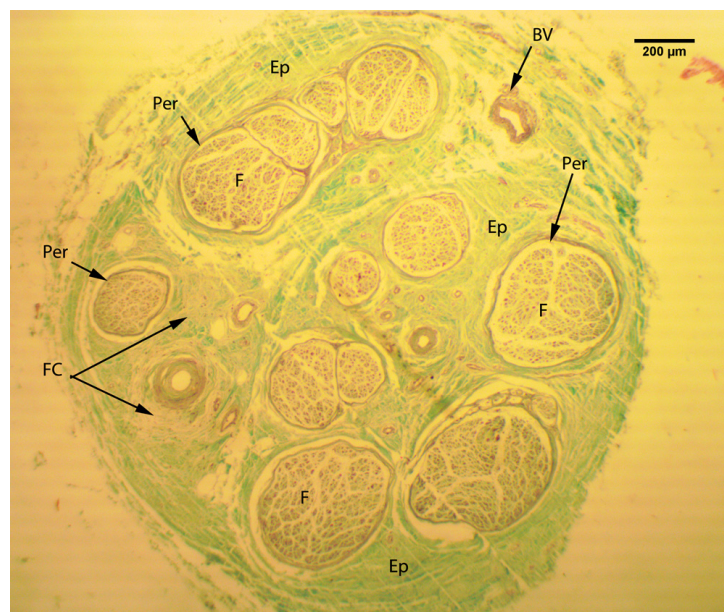
**Table 1.** Results of morphometric analysis of all investigated cases

Sample number	Sex	Age (years)	Total number of fascicles	Total cross section neural area ( $10^6/\mu\text{m}^2$ )	Total cross section epineurial area ( $10^6/\mu\text{m}^2$ )	Total cross section fascicular area ( $10^6/\mu\text{m}^2$ )	Perineurial index	Volume density of myelinated fibers (%)	Volume density of endoneurial content (%)
1	M	43	7	1.88	1.58	0.30	6.28	42.29	57.71
2	F	51	6	3.00	2.12	0.89	2.39	32.74	67.26
3	F	36	13	3.13	1.89	1.24	2.40	49.30	50.70
4	F	9	5	1.06	0.60	0.46	3.63	20.80	79.20
5	M	46	6	1.04	0.66	0.38	3.59	22.55	77.45
6	M	75	10	3.27	2.26	1.01	5.39	28.58	71.42
7	F	72	13	2.41	1.42	0.99	6.21	8.21	91.79
8	M	58	9	2.33	1.73	0.60	6.32	15.29	84.71
9	M	80	7	2.07	1.34	0.73	3.77	12.26	87.74
10	F	73	7	2.94	2.47	0.47	5.78	7.20	92.80

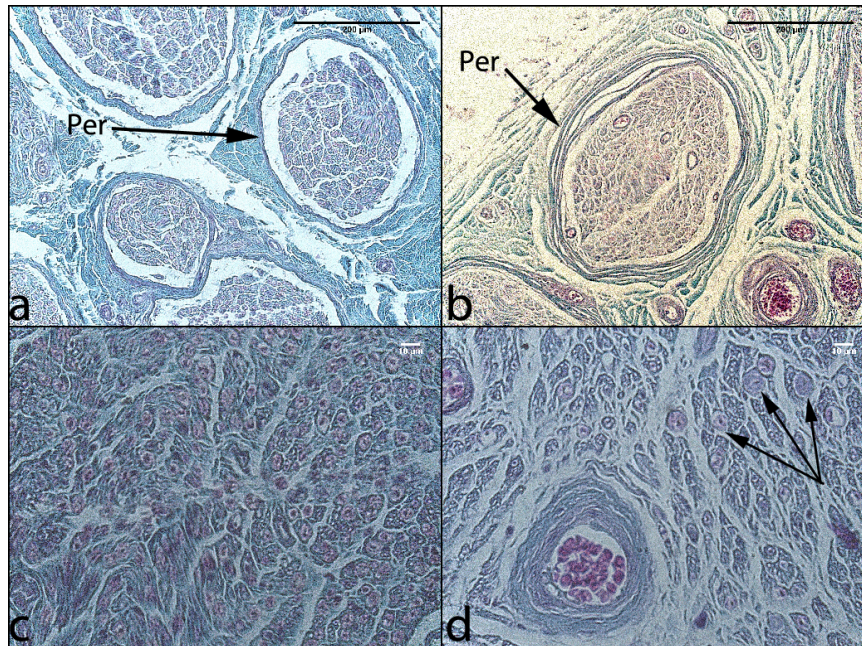
**Table 2.** Overview of the mean values of examined morphometric parameters between younger (group 1) and older group (group 2) of investigated cases with statistical significance

	Mean age (years)	Mean number of fasciculi	Total cross section neural area ( $10^6/\mu\text{m}^2$ )	Total epineurial area ( $10^6/\mu\text{m}^2$ )	Total fascicular area ( $10^6/\mu\text{m}^2$ )	Perineurial index	Volume density of myelinated axons (%)	Volume density of endoneurial content (%)
Group 1 (9-51 years)	37±17	9±3.2	2.02±1.01	1.37±0.70	0.65±0.40	3.57±1.40	33.54±12.34	66.46±12.34
Group 2 (58-80 years)	72±5	7±2.5	2.60±0.49	1.84±0.50	0.76±0.24	5.49±1.03*	14.31±8.61*	85.69±8.61*

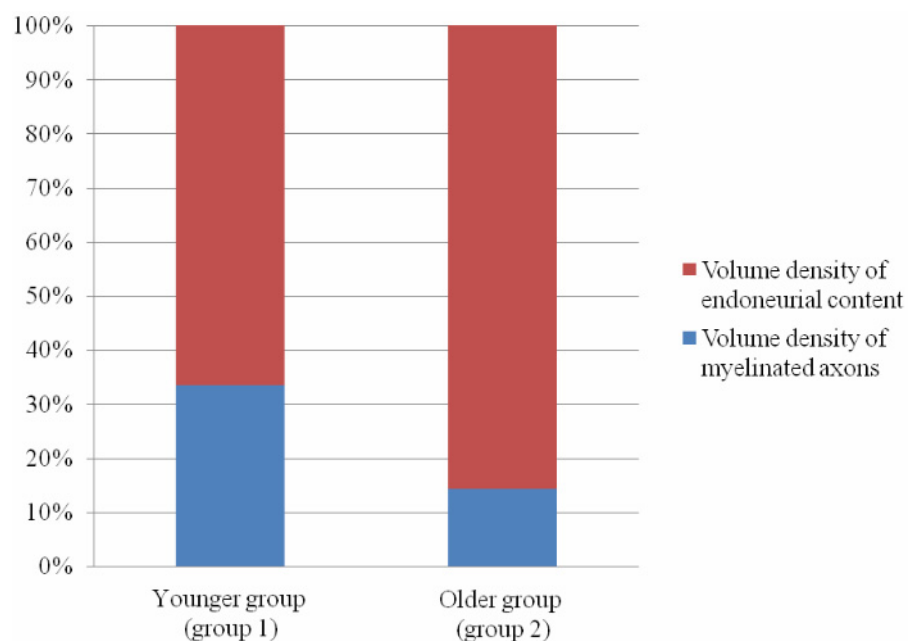
\*  $p < 0,05$



**Figure 1.** Cross section of sural nerve (female, 72 years), magnification 40x, Masson's trichrome staining, scale bar 200  $\mu\text{m}$  (Ep - epineurium; Per - perineurium; F - fasciculus; BV - blood vessel; FC - fat cells)



**Figure 2.** Fascicles of sural nerve (magnification 200x, Masson's trichrome staining, scale bar 200  $\mu$ m) of male case 43 years old (a) and female case 72 years old (b) (Per - perineurium), as well as endoneurium (magnification 630x, Masson's trichrome staining, scale bar 10  $\mu$ m of male case 43 years old (c) and 75 years old (d), with arrows pointing to the myelinated fibres stained red, while endoneurial connective tissue is stained green.



**Figure 3.** Graphic overview of volume density of myelinated axons and endoneurial content

## DISCUSSION

Both in aged humans and animals, there are age-related changes in axons that may lead to axonal degeneration, like glycogen inclusions, mitochondrial degeneration, accumulation of filaments (8). Deterioration of peripheral nerves in aging includes atrophy of large mye-

linated fibres, increased thickness of myelin sheaths and, the most common, segmental demyelination followed by consequent remyelination and myelin decompaction (9). Tanaka and Webster (10) showed that regeneration involves fewer axons because interaction between Schwann cells and sprouting axons may take longer in aging. Loss of myelin may lead to decrease in conduction velocity along axons which is additionally lowered by remyelina-

tion which is responsible for formation of new, shorter myelin internodes. This decrease extends reaction times and results in neuronal dyssynchrony which disrupts sensory and motor functions (11). Aging could be responsible for alteration in the guidance cues needed for the activation of macrophages and Schwann cells to initiate repair on the site of injury (12). Structural changes lead to the onset of mild form of neuropathy known as "senile neuropathy" (1).

There are several studies which have investigated morphologic changes in human sural nerve during aging (13-15). O' Sullivan denoted the loss of large fibers (13), as well as Jacobs and Love (14) who have also reported a reduction of unmyelinated fibers, while the study of Kanda et al. (15) did not show a significant correlation between aging and density of unmyelinated fibers. We have also detected the morphological changes of myelinated fibres in sural nerves in older cases which involved its lower density, more irregular shape and bigger presence of smaller fibres, as well as larger content of endoneurial connective tissue. Significant decrease of volume density of myelinated fibres with increase of volume density of endoneurial content indicate significant degeneration of myelinated fibres of the sural nerve with age-related endoneurial fibrosis. We may say that this result is expected, in accordance with numerous studies on human and animal nerves, but the changes in epi-, peri- and endoneurial sheath related to senile neuropathy are also the subject of recent researches.

Fascicular nerve structure (number, size and arrangement of fascicles) and variable amount of epineurium influence biomechanical resistance of the nerve, considering the fact that nerves are exposed to various grades of stretching during everyday activities. There are literature data on the fact that the peripheral nerve can tolerate stretching of 6% to 8% of its total length without morphological and functional changes, while stretching larger than 15% of its total length leads to the complete block of intraneural flow (1, 16). Fascicular structure of sural nerve did not show any difference between the investigated groups in our study. In human nerve trunks the proportion of epineurium ranges from 30% to 75% of cross section areal (2). Mean percentage of epineurium in cross section of sural nerve was 67.8% in the first group, while it was higher in the older one (70.8%), but with no statistical significance. As may be seen from Table 2, total cross section area of the sural nerve along with epineurial area shows an increase with aging, but it is not significant. The opportunities for measuring the area of the whole peripheral nerve's cross section were very rare in the past. Therefore, in the present literature there are only data for this parameter for human sural nerve (14), rat's tibial nerve (4), rat's sciatic nerve (17), and dog's trochlear nerve (16). All latter authors agreed that there is statistically insignificant increase of the whole nerve's cross section area during the aging process in all these cases. A presence of lower or higher amount of fat cells in the epineurium of su-

ral nerves of the older people which we have noticed is also described by Verheijen et al. (18), as well as Ugreovic et al. (19). Verheijen et al. cited that the presence of adipose tissue in peripheral nerves was detected during the previous researches by other authors, but its presence was underestimated. On the other hand, a larger amount of fat tissue makes microsurgical reparation of injured nerve more difficult and reduces nerve fibers regeneration in such way that they "get lost" in the rich interfascicular tissue. Barkmeier and Luschei (20) obtained significant difference between sexes in percentage of adipose tissue, whereby female subjects had it nearly 10% more than male ones.

Relevant feature of perineurial endothelial cells is the presence of numerous pinocytotic vesicles with the cavities opening on outer and inner side of the cell. On the other hand, histochemical studies showed that perineurial cells possessed wide range of phosphorylation enzymes which, along with the present pinocytotic vesicles, support the standing that perineurial diffusion barrier is metabolically active (1). Such structured perineurium does not let biological or physical particles bigger than 12 nm in diameter to leak through the barrier, so it functions as a sieve analogue to basement membrane (BM) of renal glomeruli. Ceballos et al. (4) reported that perineurium of tibial nerve of rat consists of 4-6 layers of thin perineurial cells, while the whole nerve area, as well as perineurial area, got bigger during aging, but insignificantly. Jacobs and Love (14) observed that perineurial BM in aged subjects was thicker, mainly in outer layers of perineurial sheath, in their study of qualitative and quantitative morphologic changes on human sural nerve. Inner layers were less affected. Toghi et al. (6) confirmed the linear relationship between perineurial thickness and diameter of human sural nerve fascicle and defined it as perineurial index. They have also obtained the increased perineurial index during aging and explained it by the fact that sural nerve is superficial and exposed to chronic trauma. In our study, we have also measured the perineurial index and detected significant thickening of perineurium of sural nerve fascicle with aging process. It is considered that this thickening of perineurial sheath is a consequence of thickening of BM. However, so far, only a few studies have evaluated changes of several human tissues BMs during the aging process. Previous researches which included the measurement of capillaries and seminephros tubes (21) and glomerular BMs of the kidney (22) thickness showed its significant increase during the aging process. Uspenskaia et al. (23) showed the increase of collagen type IV in cerebral micro vessels BM. Candiello et al. (24) found thickening of BM in human inner limiting membranes.

## CONCLUSION

By morphological and morphometrical analysis of fascicular structure and connective tissue sheaths of sural nerve during aging we detected significant thickening

of perineurial sheath with endoneurial fibrosis. Further investigations should aim at quantitative analysis of the extracellular matrix components of perineurium and endoneurium, such as type III, IV, VI collagen, fibronectin, laminin and others.

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## ANALIZA FASCIKULARNE STRUKTURE I VEZIVNOTKIVNIH OMOTAČA SURALNOG NERVA SA STARENJEM

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### Sažetak

Cilj našeg istraživanja bio je da analiziramo promene vezivnotkivnih omotača epi-, peri- i endoneurijuma suralnog nerva sa starenjem.

Ispitivanje je izvedeno na uzorku suralnog nerva 10 slučajeva starosti 9-80 godina. Uzorci su ukalupljeni u parafinu po standardnim procedurama, nakon čega su pravljene poprečni preseki nervnog stabla debljine 5  $\mu\text{m}$  i bojeni po Masonovom trihromnom bojenju. Nakon morfološke analize fascikularne strukture i vezivnih omotača nerva, izvršena je morfometrijska analiza korišćenjem programa za analizu digitalne slike ImageJ. Na svakom ispitivanom uzorku mereni su ukupna neuralna, epineurijalna i fascikularna area na poprečnom preseku nerva, srednja vrednost perineurijalnog indeksa, volumenska gustina mijelinizovanih aksona i endoneurijalnog sadržaja. Za testiranje razlike među srednjim vrednostima na statističku značajnost korišćen je Studentov T-test za male nezavisne uzorke.

Broj fascikulusa kretao se od 5 do 13, pri čemu je većina imala manje od 10 fascikulusa. Fascikularna struktura, koja se odnosi na broj fascikulusa i odnos epineurijalne i fascikularne aree na preseku nerva, nije pokazala značajne promene sa starenjem. Odnos debljine perineurijuma i veličine fascikulusa statistički značajno raste u starijoj ispitivanoj grupi ( $p < 0,05$ ). Kod slučajeva starije starosne grupe mijelinska vlakna su bila manjeg dijametra, nepravilnijeg oblika i znatno ređa. Kvantitativna analiza pokazala je statistički značajan pad volumenske gustine mijelinskih vlakana u starijoj grupi.

Primenjenim metodama istraživanja utvrdili smo zadebljanje perineurijumskog omotača suralnog nerva sa starenjem, kao i endoneurijalnu fibrozu. Buduća ispitivanja starosnih promena treba usmeriti ka analizi komponenata ekstracelularnog matriksa unutar perineurijuma i endoneurijuma.

**Ključne reči:** suralni nerv, starenje, degeneracija, perineurijum, endoneurijalni fluid