The Effect of Exogenous Melatonin on the Extrrafascicular Connective Tissue in Transected Rat Sciatic Nerve

Esad Ćosović¹, Zakira Mornjaković¹, Selma Aličebelić¹, Dina Kapić¹, Maida Šahinović¹, Almira Lujinović², Višnja Muzika¹, Samra Ćustović¹

Abstract
Previous studies linking the effect of certain pharmacological agents with the status of connective tissue and nerve fiber regeneration after traumatic transection were focused mainly on the proximal nerve stump. In our study, qualitative and quantitative histological analysis of the proximal and the distal nerve stump were done. Male Wistar rats underwent transection and excision of an 8-mm nerve segment of the left sciatic nerve. The vehiculum group of animals (n=7) was administered with 5% ethanol in Ringer solution (vehiculum), while the melatonin group (n=10) received 30mg/kg of melatonin dissolved in vehiculum, daily, intraperitoneally (i.p.) for 14 consecutive days. Then, intravital excision of the marginal zone of the proximal and distal nerve stump was performed and the samples were further processed for qualitative photomicroscopic and stereological analysis. Macroscopic and microscopic examinations of both nerve stumps showed absent or slight stump thickening in the melatonin group compared to the vehiculum group of animals, which is the result of reduced connective tissue proliferation. The mean epineurial volume density of the proximal nerve stump was statistically significantly lower (p=0,003) in the melatonin (0,36) than in the vehiculum group of animals (0,51). The difference in mean epineurial volume density of the distal stump was also statistically significant (p=0,039) with 0,33 in melatonin and 0,46 in the vehiculum group. Our study revealed that the administration of exogenous melatonin was effective in suppression of trauma-caused extrarfascicular connective tissue proliferation in neuroma of the proximal nerve stump as well as fibroma formation in the distal nerve stump.

Keywords
rat — sciatic nerve — nerve injuries — melatonin — histology

¹Department of Histology and Embryology, Faculty of Medicine, University of Sarajevo, Sarajevo, B&H
²Department of Anatomy, Faculty of Medicine, University of Sarajevo, Sarajevo, B&H
*Corresponding author:esad.cosovic@mf.unsa.ba

Introduction
Accumulation of connective tissue in the proximal stump after nerve transection is one of the factors that inhibit successful nerve regeneration. It forms a mechanical barrier for axonal growth and impairs its morphofunctional recovery. Thus, reduced collagen deposition in both proximal and distal nerve stumps could result in a nerve sparing resection and consequently diminished tension in the postponed interventions. Intrafascicular deposition of collagen after nerve injury is more prominent in non-myelinated than in predominantly myelinated nerve fibers. Also, it depends on the type of injury and is particularly intensive after nerve transection (11, 12, 15, 29).

The connective tissue accumulation is most excessive in the 2,5-mm segment proximal and distal to the transection site (2,21). The finding of neuroma indicates axonotmesis and neurotmesis while the neuroma shape and its localization are clinically significant. Nowadays, there is an increased interest for the studies on the prevention of connective tissue accumulation in the nerve stump.

Studies on the effect of melatonin are particularly interesting. Recent studies analyzed nerve recovery after transection in pinealectomized rats. The results show that pinealectomy in rats is associated with increased collagen content in the wound granulation tissue. Also, the administration of exogenous melatonin reduces the amount of total and soluble collagen in a dose-dependent manner (5, 8, 9, 16, 28).

A massive neuroma is formed after pinealectomy and neurectomy of sciatic nerves in rats. Microscopically, massive proliferation of connective tissue and Schwann cells as well as numerous microfascicles oriented in various directions are present. The recovery of the injured nerve depends on the regenerative potential of Schwann cells and connective tissue accumulation (7, 11, 12). The reduction of connective tissue and simultaneous increase in nerve tissue content in the proximal stump are evident in pinealectomized rats treated with melatonin (0,3 mg/kg, subcutaneous injection) after sciatic nerve transection (27). Melatonin administered in dose of 50 mg/kg shows strong neuroprotective effect in the condition of blunt sciatic nerve injury in rats, while the dose of 10 mg/kg diminishes trauma-associated


myelin disintegration and reactive axonal changes (23).

Melatonin inhibits the synthesis of TGF-β1 and consequently impairs its promoting effects on collagen deposition. The result of this action is an indirect protective effect of melatonin on nerve regeneration processes at the site of surgical transection (28). Also, melatonin has antioxidant properties and serves as a free-radical scavenger in vitro and in vivo. It is a potent stimulator of endogenous antioxidants including superoxide dismutase, glutathione peroxidase and glutathione reductase. Melatonin inhibits neutrophils infiltration after tissue reperfusion (4, 6, 18, 26). Exogenous melatonin has proved its potent antiinflammatory and antioxidant properties in induced liver fibrosis in rats (5, 25).

The main focus of recent experimental studies on the effect of melatonin on nerve regeneration after transaction was the histological assessment of the proximal nerve stump, while the results of melatonin effect on the distal stump are still missing. The aim of this study was to describe and compare the morphological changes in the proximal and the distal stump of the transected rat sciatic nerve after melatonin administration. The results of such an approach could contribute to a better understanding of the regenerative capacities of injured nerves.

### Material and Methods

**Animals.** This study was performed using two-month-old male Wistar rats. All the procedures were in accordance with the animal protection laws and guidelines and were approved by the local institutional review board. The animals were housed in standardized laboratory conditions (12-hour light-dark cycles, temperature 23±2°C). The animals were fed regular chow and drank water ad libitum.

**Experimental design.** After one week of acclimatization, the animals were randomly assigned into two groups: vehiculum (n=7) and melatonin group (n=10). The animals were anesthetized with ketamine (50 mg/kg, i.p.) and the operating field was prepared in the left gluteal region. A longitudinal skin incision was made and the muscles were dissected in order to access the sciatic nerve. After the sciatic nerve was clearly exposed, transection was performed and an 8-mm nerve segment was excised. All animals were postoperatively monitored. The animals from the vehiculum group were treated with 5% ethanol in Ringer solution i.p. for 14 consecutive days. At the same time, the rats from the melatonin group were administered i.p. with melatonin (Sigma-Aldrich, St. Louis, MO, USA) at the dose of 30mg/kg dissolved in 5% ethanol in Ringer solution (2.5 mg/mL). The application was performed every day at the same time (18-19 h).

At the end of the experiment, all animals were anesthetized using ketamine (50mg/kg, i.p.) and the sciatic nerve was surgically exposed in order to perform the semi-quantitative analysis of stump macroscopic findings. The adhesion intensity and the extensiveness of the neuroma in the proximal and fibroma in the distal nerve stump were estimated according to Turgut et al. (27). The changes were graded as: absent (−), slight (+), moderate (++) and severe (+++).

**Histological analysis.** After the gross examination of the nerve stumps, animals were sacrificed using ether anesthesia. Samples, 10 mm sciatic nerve segments, were taken from the marginal zones of both stumps, fixed in 10% buffered formalin and embedded in paraffin blocks. Five micrometer thick serial transverse sections were cut and stained with hematoxylin and eosin (HE), Azan and periodic acid-Schiff (PAS). Qualitative and stereological analysis were performed according to previously described guidelines (14, 17, 19, 20) using light microscopy and a modular software for interactive image processing and analysis (Ellipse, Version 2.0, 8, 1).

**Statistical analysis.** Values with normal distribution were expressed as mean ± standard deviation and statistically significant differences between groups were determined with Student’s t test. P-values less than 0.05 were considered statistically significant. Statistical calculations were performed using SPSS 13 software (version 13.0, SPSS Inc, Chicago, Illinois, USA) and Microsoft Excel (version 11.0, Microsoft Corporation, Redmond, WA, USA).

### Results

**Gross observation**

There was no mortality in any of the groups. No clinical or local signs of infection were noted. The animals tolerated all the procedures well, including surgical excisions and application of melatonin or vehiculum.

The results of the semiquantitative analysis regarding thickening and adhesion of proximal and distal nerve stumps in both groups are presented in Table 1. Thickening was more prominent in the vehiculum group, and presented in 100% of proximal and 71% of distal nerve stumps. In contrast, thickening was absent in 80% of proximal and 70% of distal stumps in the melatonin group. Also, in the melatonin group adhesion of both nerve stumps to the paraneurial tissue was less frequent. The differences in macroscopic features of the proximal and distal nerve stumps between the two groups were apparent as shown in Figure 1.

**Table 1.** The results of the semiquantitative analysis of proximal and distal stumps after the sciatic nerve transection in both groups

<table>
<thead>
<tr>
<th>parameter</th>
<th>Vehiculum (n=7)</th>
<th>Melatonin (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>thickening</td>
<td>proximal stump</td>
<td>proximal stump</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>6</td>
<td>2</td>
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<td>++</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>adhesion</td>
<td>distal stump</td>
<td>distal stump</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>3</td>
</tr>
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<td>+</td>
<td>6</td>
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<tr>
<td>++</td>
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<td>1</td>
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<td>+++</td>
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<td>0</td>
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</tbody>
</table>

- absent; + slight; ++ moderate; +++ severe.
Figure 1. Macroscopical appearance of the proximal and distal nerve stump. A Proximal nerve stump in vehiculum group is adhered to the surrounding tissue by its mesoneurium sheet, and neuroma causes the clubbing deformation (arrows) approximately doubled in size of the unaltered part; B Proximal stump in melatonin group is slightly adhered to the paraneural connective tissue through its mesoneurial sheet; C Distal nerve stump in vehiculum group shows moderate adhesion to adjacent tissue while clearly visible thickening (arrows) extends even below the transection site; D Adhesion is obviously absent and stump thickness is not altered in the distal stump of melatonin group.

**Qualitative histological study**

**Vehiculum group – proximal nerve stump**

In the vehiculum group of animals, the epifascicular epineurium of the proximal nerve stump was thickened and composed of irregular dense connective tissue with indistinctive border to the perineurium. Its main component was collagen fibers arranged in fascicles, which surrounded smaller or larger areas of extrafascicularly positioned Schwann cells. Perineural lamellae were disintegrated without distinct demarcation to the adjacent interfascicular and extrafascicular areas. Sporadically, some fascicular structures were herniated into the epineurium (Fig. 2A).

**Vehiculum group – distal nerve stump**

The analysis of the distal nerve stump showed proliferation of the epineurial connective tissue, in which collagen bundles were abundant and oriented in various directions. Adjacent blood vessels were dilated. There was an adhesion of the surrounding connective tissue to the nerve epineurium. The epineurial tissue pervaded the perineurial lamellae, thus resulting in loss of distinction between these two compartments (Fig. 2C).

**Melatonin group – proximal nerve stump**

Qualitative histological study of the proximal nerve stump in the melatonin group revealed only a mild proliferation of the peripheral part of the epineurium and slightly intense proliferation of the epineurium adjacent to the perineurium. This tissue was hypercellular with domination of fibroblasts and abundant extracellular matrix. Blood vessels in these areas were dilated. The demarcation zone of the perineurium to the adjacent epifascicular epineurium was uneven, thus making the border between epi- and perineurium in some areas indistinctive (Fig. 2B).

**Melatonin group – distal nerve stump**

Qualitative histological study of the distal nerve stump in the melatonin group showed more compact and cellular epineurium with domination of fibroblasts, while collagen fibers were densely packed and intensely stained with eosin. Blood vessels in the epineurium and the perineurium were dilated. Perineurial layers were disintegrated in some areas and they were also indistinctly bordered towards the adjacent epifascicular epineurium. A clear distinction between fascicles, epi- and interfascicular epineurium was not evident. The perineurium was well demarcated towards the intrafascicular area (Fig.2D).
The Effect of Exogenous Melatonin

Figure 2. Photomicrographs of proximal and distal stump of transected sciatic nerve. A Vehiculum, proximal stump (Azan, 100x); B Melatonin, proximal stump (PAS, 100x); C Vehiculum, distal stump (Azan, 100x); D Melatonin, distal stump (HE, 40x).

Table 2. Volume density (Vv) of the epineurium in the proximal and distal nerve stump/mm^0

<table>
<thead>
<tr>
<th>Epineurial volume density</th>
<th>Proximal stump</th>
<th>Distal stump</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vehiculum</td>
<td>Melatonin</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Mean</td>
<td>0.51</td>
<td>0.36</td>
</tr>
<tr>
<td>SD^a</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>95% CI b</td>
<td>0.43-0.58</td>
<td>0.30-0.43</td>
</tr>
<tr>
<td>Range (min-max)</td>
<td>0.41-0.64</td>
<td>0.28-0.54</td>
</tr>
<tr>
<td>p value</td>
<td>0.003*</td>
<td></td>
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</tbody>
</table>

Variables show normal distribution and t-test was used for statistical testing; ^a standard deviation; ^b confidence interval
*Statistical significance level was set as p<0.05

Stereological analysis

The differences of mean epineurial volume density between the vehiculum and the melatonin group were statistically significant for both proximal (p=0.003) and distal nerve stumps (p=0.039). Results of the stereological analysis are presented in Table 2.

Discussion and conclusion

The macroscopical semiquantitative analysis of the proximal nerve stump in the melatonin group of animals showed only a slight thickening in 20% of examined nerve stumps, while in 80% of nerves thickening could not be identified by gross observation. In the vehiculum group, all nerve stumps were thickened with 86% graded as slightly and
14% as moderately thickened. Apart from reducing neuroma formation, application of exogenous melatonin lowers the possibility of stump adhesion to the paraneurial tissue. In our study, a slight adhesion was found only in 40% of the proximal nerve stumps in the melatonin group, and 86% in the vehiculum group. Observed thickening of proximal nerve stumps in the vehiculum group was the result of epineurial thickening with predominant collagen fibers and dilated blood vessels. Reactive changes in the extrafascicular tissue were of the same quality but less prominent than those found in the vehiculum group.

The findings of neuroma and intense connective tissue proliferation in the proximal nerve stump in the vehiculum group, and less intensive proliferation in the melatonin group were quantified by stereology and compared using statistical analysis. There was a statistically significantly lower (p=0.003) mean volume density of the epineurium in the melatonin group (Vv=0.36±0.086) compared to the vehiculum group (Vv=0.51±0.08). Because transection of peripheral nerves is accompanied with inflammatory events and regeneration, observed thickening in the proximal stump of all nerves in the vehiculum group can be explained by intensive proliferation of extrafascicular tissue. Absence of proliferation in 80% of proximal nerve stumps in the melatonin group can be explained by increased regeneration and suppression of connective tissue proliferation. Similarly, Fischer et al. (13) described a prominent neuroma of 2 mm in diameter in rats 30 days after transection, and explained the increased formation of neural tissue as a result of greater density of axons in the sciatic nerve model. The same authors used light and electron microscopy to show an increase in number of axons per unit area. The ability of melatonin to promote axonal growth and suppress collagen production at physiological levels was studied by Turgut et al. (27). They used a rat sciatic nerve transection model and described macroscopic and microscopic findings in the following three groups: transection, transection and sham-pinealectomy, and transection and pinealectomy with consecutive application of exogenous melatonin (0.3 mg/kg) for four weeks. Alterations described in the transection and the transection and sham-pinealectomy group were more pronounced than our findings in the vehiculum group. This can be explained by a difference in duration of the experiment. In contrast to Turgut et al. (27), we found a less prominent neuroma in the melatonin group because of a relatively high dose of melatonin administered in our study. Also, Atik et al. (1) reported lack of beneficial effects of melatonin at physiological doses thus proposing higher doses and longer duration of treatment. Melatonin of extra-pineal origin must be considered in the interpretation of physiological melatonin levels in pinealectomy (24). Thus, we find it justified to consider melatonin as a potential therapeutic strategy for peripheral nerve injury (3). Also, the protective role of melatonin is related to its activity as a direct free radical scavenger, and its indirect antioxidative effect (22).

A more detailed insight in the total regenerative capacity of damaged nerves can be gained by evaluation of not only the proximal but also the distal stump of the transected nerve. The semiquantitative analysis of the macroscopically observed thickening of the distal nerve stump showed a slight thickening in 30% of nerves in the melatonin group, while 70% of them showed no thickening at all. Thickening of the distal stump was more evident in the vehiculum group (71% nerves). Status assessment of the distal nerve stump after transection showed a beneficial effect of melatonin application because slight adhesion was found in only 20% of nerves in the melatonin group. In the vehiculum group, slight adhesion was found in 43% and moderate in 14% of nerves. Similar to the processes in the other histological compartments, the wound healing process in damaged nerves is composed of different phases: inflammation, granulation, remodeling and scarring. Our qualitative histological analysis of the distal nerve stump in the vehiculum group showed alterations in the connective tissue - vascular compartment in the form of an intensive proliferation of the epineuric connective tissue, with evident domination of collagen bundles oriented in various directions. Also, dilated blood vessels and areas of adherent surrounding connective tissue were present. It is assumed that melatonin shows indirect effects on the total regenerative capacity of the sciatic nerve after surgical transection through reduction of synthesis of TGF-β 1 and collagen type I and increase in bFGF (21,28). Alterations in connective tissue - vascular areas in the distal stump were similar but less pronounced in the melatonin group compared to the vehiculum group. The epineurium was hypercellular with abundant fibroblasts, dominant collagen fibers and slightly dilated blood vessels. Macroscopic findings of glioma and connective tissue accumulation in the distal stump of the vehiculum group and its less frequent finding in the melatonin group were quantified by stereology, and the observed differences were confirmed by statistical analysis. There was a significantly lower (p=0.039) mean volume density of the epineurium in the melatonin group (Vv=0.33±0.01) compared to the vehiculum group (Vv=0.46±0.03). Our results are in accordance with those found by Ngeow et al. (21), and Eather et al. (10). Those studies, using a model of sciatic nerve transection in rats, showed that accumulation of connective tissue was more extensive in nerve segments 2.5 mm proximal and distal to the transection site (10,22).

Nerve transection results in the formation of a neuroma in the proximal and a fibroma in the distal nerve stump, both accompanied with adhesion to the paraneurial connective tissue. High doses of exogenous melatonin in both proximal and distal nerve stumps suppress collagen synthesis in all nerve sheaths, thus reducing the mean epineurial volume density.

### References

Utjecaj egzogenog melatonin na ekstrafascikularno vezivno tkivo u presječenom bedrenom nervu pacova

Sažetak

Uvod i ciljevi

Nakon traumatske transekcije nerva, akumulacija vezivnog tkiva na proksimalnom okrajku je jedan od negativnih faktora koji koče regeneraciju nerva, s obzirom da proliješirano vezivo predstavlja mehaničku barijeru za rast aksona, a time i uspješan morfofunkcionalni oporavak nerva. Istraživanja prevencije akumulacije vezivnog tkiva na okrajcima presječenog nerva intenzivirana su posljednjih godina, što se posebno odnosi na upotrebu melatoninina. Dosadašnja istraživanja utjecaja nekih od farmakoloških agenata na status vezivnog tkiva i nervnih vlakana okrajaka nerva nakon traumatske transekcije bila su fokusirana uglavnom na proksimalni okrajak nerva. Smatrajući da je u uvjetima aplikacije melatoninina neophodno procijeniti uporedo status i proksimalnog i distalnog okrajka presječenog nerva, kako bi se dobio kompletiji uvid u ukupne regenerativne kapacitete oštećenog nerva, poduzeli smo ovo istraživanje.

Materijal i metode

U ovom istraživanju, izvršili smo kvalitativnu i kvantitativnu histološku analizu oba okrajka presječenog ishijadičnog nerva. U eksperimentu smo koristili mužjake Wistar pacova starosti dva mjeseca. Kontrolnoj grupi (n=7) smo nakon transekcije lijevog ishijadičnog nerva i ekszizije segmenta u dužini 8mm jednom dnevno i.p. aplikirali vehikulum (5% etanol u Ringerovoj otopini). Eksperimentalna grupa (n=10) je tretirana rastvorom melatoninina u dozi od 30mg/kg tjelesne mase, jednom dnevno u periodu od 8 dana, koliko su tretirane i kontrolne životinje. Potom smo izvršili intravitalnu eksziziju graničnih zona okrajaka nerva i isječke histotehnološki pripremili za kvalitativnu analizu. Serijski rezovi, pojedinačne debljine 5µm, bojeni su metodama: HE, Azan PAS.

Statistička analiza je podrazumijevала analizu simetričnosti kontinuiranih varijabli i normalnost njihove raspodjele. Pošto je raspodjela kontinuiranih varijabli bila simetrična, koristili smo aritmetičku sredinu (X), standardnu devijaciju (SD) i standardnu grešku srednje vrijednosti (SEM) za prikaz srednje vrijednosti i mjera raspršenja, a za poredjenu tih varijabli, koristili smo parametrijski test (Studentov t-test). Vrijednosti p<0,05 smatrali smo statistički značajnim. Statističku analizu dobivenih podataka koristili smo programski paket SPSS for Windows (verzija 13.0, SPSS Inc, Chicago, Illinois, SAD) i Microsoft Excel.

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Rezultati i interpretacija

Makroskopski i mikroskopski nalaz oba okrajka presječenog nerva pokazao je odsustvo ili blagi stepen zadebljanja okrajaka kod eksperimentalne u odnosu na kontrolnu grupu, zbog manje proliferacije veziva. Stereološkom analizom u proksimalnom okrajku ustanovljena je značajno manja volumenska gustoća epineurijuma eksperimentalne grupe (0,36) u odnosu na kontrolnu grupu (0,51), a identično stanje evidentirano je i u distalnom okrajku s vrijednostima volumenske gustoće epineurijuma 0,33 u eksperimentalnoj i 0,46 u kontrolnoj grupi. Slijedom semikvantitativne analize makroskopski evidentiranih zadebljanja proksimalnog okrajka naših eksperimentalnih životinja, utvrdili smo kod 20% nerava neznatno zadebljanje u proksimalnom okrajku, dok čak 80% nerava nije imalo zadebljanja. U kontrolnoj grupi evidentirali smo zadebljanje okrajaka svih nerava, i to kod 86% nerava neznatno i kod 14% nerava umjerenje zadebljanja. Također smo evidentirali neznatna zadebljanja distalnog okrajka kod 30% nerava u eksperimentalnoj grupi, dok čak 70% nerava nije imalo zadebljanja. Značno češće smo evidentirali zadebljanje distalnog okrajka u kontrolnoj grupi, i to kod 71% nerava. Aplikacija egzogenog melatoninina reducira stvaranje neuroma, a također smanjuje i učestalost adhezije okrajaka za paraneuralno vezivo. Ovo je potvrdio naš makroskopski nalaz neznatne adhezije proksimalnog okrajka za okolno paraneuralno vezivo kod 40% nerava životinja eksperimentalne grupe u odnosu na znatno veći procenat (86% nerava) u kontrolnoj grupi. Benčef aplikacije melatoninina na status distalnog okrajka nerva nakon transekcije pokazao je i makroskopski nalaz neznatne adhezije kod 20% nerava u eksperimentalnoj grupi u odnosu na učestalije prisutnu adheziju neznatnog stepena kod 43% nerava te umjerenog stepena kod 14% nerava u kontrolnoj grupi. Naši rezultati pokazuju efektivnost egzogenog melatoninina u pravcu značajne supresije traumom provocirane proliferacije ekstrafascikularnog veziva kod formiranja neuroma u proksimalnom i glioma iz distalnog okrajka nerva.

Glavni zaključci

Trajanceka rezultirala formiranjem neuroma u proksimalnom i glioma iz distalnog okrajka nerva uz adheziju za paraneuralno vezivo. U oba okrajka nerva, visoka doza egzogenog melatoninina suprimira sintezu kolageni svih omotnih sustava, pri čemu značajno smanjuje srednju vrijednost volumenske gustoće epineurijuma.