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Time-Dependent Proregenerative Effects of Exogenous Melatonin on the Transected Sciatic Nerve

Efectos Proregenerativos Dependientes del Tiempo de la Melatonina Exógena en el Nervio Ciático Seccionado

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SUMMARY: Microsurgical procedures are the treatment of choice of peripheral nerve injuries, but often fail to reach full functional recovery. Melatonin has neuroprotective actions and might be used as a possible proregenerative pharmacological support. Therefore, the aim of this study was to analyze the time-dependence of the neuroprotective effect of melatonin on the overall fascicular structures of both ends of the transected nerve. Sciatic nerve transection was performed in 34 adult male Wistar rats divided in four groups: two vehicle groups (N=7) treated intraperitoneally for 7 (V7) or 21 (V21) consecutive days with vehicle (5 % ethanol in Ringer solution) and two melatonin groups (N=10) administered intraperitoneally 30 mg/kg of melatonin for 7 (M7) or 21 (M21) consecutive days. At the end of the experiment, proximal stump neuroma and distal stump fibroma were excised and processed for qualitative and quantitative histological analysis. Intrafascicular neural structures were better preserved and the collagen deposition was reduced in the melatonin treated groups than in the vehicle groups. Myelin sheath regeneration observed through its thickness measurement was statistically significantly ($p<0,05$) more pronounced in the M21 (1,23±0,18 μm) vs. V21 group (0,98±0,13 μm). The mean volume density of the endoneurium was lower in both melatonin treated groups in comparison to the matching vehicle treated groups. Although not statistically different, the endoneural tube diameter was larger in both melatonin groups vs. vehicle groups, and the effect of melatonin was more pronounced after 21 days (24,97 % increase) vs. 7 days of melatonin treatment (18,8 % increase). Melatonin exerts a time-dependent proregenerative effect on nerve fibers in the proximal stump and an anti-scarring effect in both stumps.

KEY WORDS: Nerve transection; Melatonin; Myelin sheath; Endoneurium.

INTRODUCTION

Intrafascicular deposition of connective tissue, as the result of peripheral nerve injuries, creates a mechanical barrier and often completely prevents the passage of the regenerating axon towards the effector organ. In particular, peripheral nerve transection results in intense inflammatory-regenerative changes and connective tissue deposition at the nerve stumps. Collagen deposition is more intense, and axotomy results in lower mitotic activity of Schwann cells in damaged nerves with predominantly unmyelinated nerve fibers (Eather & Pollock, 1987, 1988). Diffuse collagen deposition, which extends through the distal segment, leads to reduction of the endoneural tube lumen and significantly slows down the passage of axons through that area (Rosenbluth, 1998). Minimizing connective tissue deposition at the nerve endings reduces aberrant axonal growth and accelerates axonal regeneration (Yick *et al*., 2003; Graham *et al*., 2007). The application of experimental pharmacological agents that reduce scar formation and promote nerve regeneration is a good auxiliary measure in the microsurgical reconstructive procedures. There are numerous studies indicating the neuroprotective effect of melatonin at physiological and pharmacological doses (Turgut *et al*. 2005; Altunkaynak *et al*., 2018; Majidiniaa *et al*., 2018; Tan *et al*., 2020). Most researchers believe that melatonin has a protective effect at a dose of 1 to 50 mg / kg (Rogério *et al*., 2002; Tan *et al*., 2020). The protective and proregenerative effects of melatonin may be associated with its direct effect on free radicals or with its indirect action in

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preventing a decrease in the levels of antioxidant enzymes in damaged nerves (Rogério *et al*., 2002; Sayan *et al*., 2004; Reiter *et al*., 2005; Chang *et al*., 2008).

Also, the proregenerative activity of melatonin is mediated by MT1 receptors on axons and Schwann cells (Stazi *et al*., 2021). It is necessary to consider the influence of melatonin on the regenerative potential of the injured nerve through its influence on nervous tissue, but also on connective tissue and blood vessels. In addition to its direct neuroprotective effect, melatonin reduces endoneural connective tissue deposition and preserves the endoneural tubes in proximal and distal stumps of severely injured nerves, and has in that way beneficial effects on the nerve fascicular organization and regeneration.

MATERIAL AND METHOD

Animals. This experimental study was carried out on adult male Wistar rats. The rats were kept under standardized laboratory conditions with free access to food and water, a constant temperature (23 ± 2 °), and a 12h light/12h dark cycle. All the animal experiments were carried out in full accordance with national and international legal regulations and principles of conducting experiments on experimental animals, and were approved by the Institutional Ethics Committee (Protocol Number: 29-NS-3953-09).

Experimental design. After a one-week adaptation period, the animals were randomly divided into four groups: two vehicle groups (7 animals per group) and two melatonin groups (10 animals per group). All animals were deeply anesthetized with ketamine (50 mg / kg, i.p.) and the operative field in the left gluteal region was prepared. A longitudinal skin incision was made and the gluteal muscle was surgically split to expose the sciatic nerve. After clear visualization of the nerve, transection followed by excision of an 8-mm long segment was done. The wound was subsequently closed in layers and treated with povidone iodine and 70 % alcohol. Rats in the vehicle groups were injected (intraperitoneally, once a day) with 5 % ethanol in Ringer's solution for 7 days (V7) or 21 days (V21). In the melatonin groups, melatonin (Sigma-Aldrich, St. Louis, MO, USA) in a dose of 30 mg/kg, dissolved in 5 % ethanol in Ringer's solution, was administered (intraperitoneally, once a day) for 7 days (M7) or 21 days (M21). The application was carried out every day at the same time (6-7 p.m.). At the end of the experiment, the animals were anesthetized with ketamine (50 mg/kg, i.p.), the sciatic nerve was surgically exposed and a macroscopic observation of the nerve stumps was performed.

Histological analysis. Following macroscopic observation of the nerve stumps, the animals were sacrificed with an additional dose of anaesthetics and the proximal marginal zone (neuroma) and distal marginal zone (fibroma) of the transected nerve were excised. Half of the neuroma was isolated, fixed in McDowell's fixative and further prepared for transmission electron microscopic analysis. The remaining samples were fixed in 10 % buffered formalin, embedded in paraffin, cut into 5 micrometer-thick sections and stained with HE, Azan and PAS for analysis by light microscopy. Qualitative histological analysis followed by quantitative analysis were performed according to the instructions given earlier (Genua *et al*., 2001) and using the image processing and analysis software-ELLIPSE (Version 2, 0, 8, 1). Volume density of the proximal nerve stump endoneurium (indicator of collagen deposition), myelin sheath thickness in the proximal nerve stump (indicator of myelin sheath regeneration) and distal nerve stump endoneurial tube diameter (to follow the possible trajectory of regenerating axons) were measured.

Statistical analysis. Values are expressed as arithmetic mean ± standard deviation. SPSS for Windows (version 13.0, SPSS Inc, Chicago, Illinois, USA) and Microsoft Excel (version 11. Microsoft Corporation, Redmond, WA, USA) were used to statistically assess the obtained data, and Student's t test was conducted for comparison. P values less than 0.05 were considered statistically significant.

RESULTS

Qualitative histological analysis. (Figs. 1 to 3)

V7 group. The endoneurial space was edematous containing finely dispersed collagen fibers, and an increased number of Schwann cells, fibroblasts, and macrophages (Fig. 1). In large myelinated nerve fibers, decomposition, delamelation and fragmentation of the myelin sheath were observed. In contrast, the myelin sheath of thinner nerve fibers was relatively intact and surrounded by a discrete layer of Schwann cell cytoplasm and a slightly deformed, but continuous and prominent external lamina. The unmyelinated axons were relatively preserved (Fig. 2). In the distal stump, intrafascicularly, hypercellularity was observed in all areas. Blood vessels were dilated and lined by a hypertrophic endothelium. The collagen fibers of the endoneurium were heterogeneously arranged. The endoneural tubes had relatively regular lumina and were empty or contained remnants of degenerating nerve fibers and associated Schwann cells (Fig. 3).

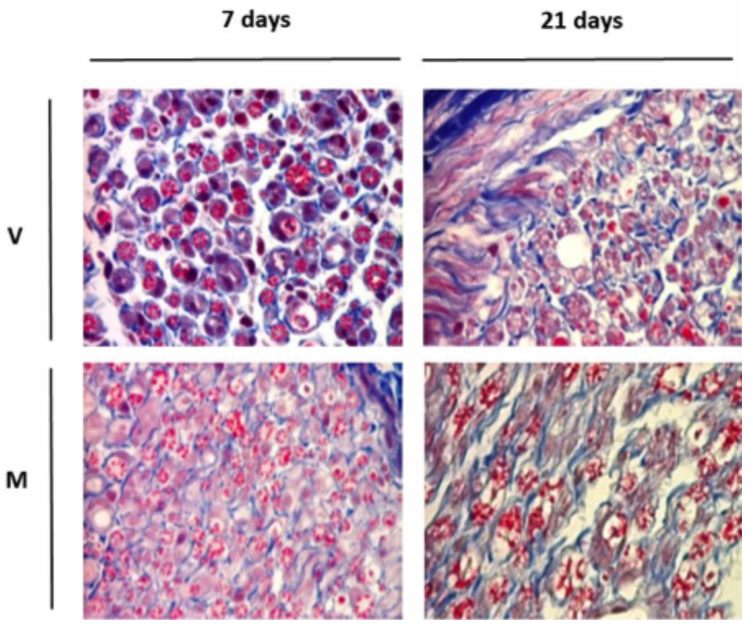

Fig. 1. Photomicrographs of the proximal nerve stump in the vehicle (V) and melatonin (M) group at 7 and 21 days postoperatively, 400x (Azan).

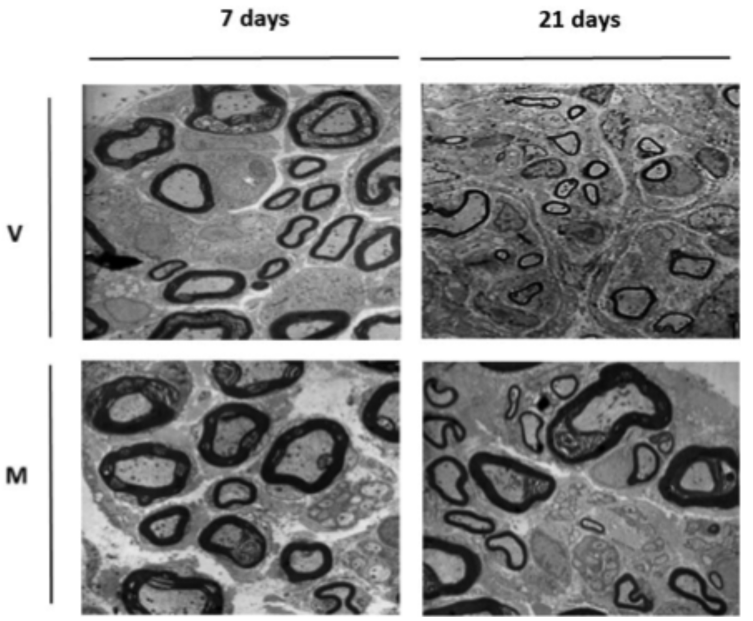

Fig. 2. Transmission electron micrographs of the proximal nerve stump in the vehicle (V) and melatonin (M) group at 7 and 21 days postoperatively, 1600x.

M7 group. The endoneural space was narrow, homogenous, hyper cellular with numerous Schwann cells. (Fig. 1). Thin myelinated and unmyelinated nerve fibers were relatively preserved, while large myelinated nerve fibers were showing different degrees of degeneration. Regenerating axons were ensheathed by condensed domains of Schwann cell cytoplasm. The endoneurial collagen fibers were organized in form of a narrow and regular belt, and separated from the Schwann cell by a well-defined, prominent and continuous external lamina (Fig. 2). In the distal nerve stump, intrafascicularly, hyper cellularity was observed in all areas. Blood vessels were dilated with a prominent endothelium. The collagen fibers were relatively uniformly arranged and formed a clear margin of the endoneurial tubes. The lumina of the endoneurial tubes were empty or contained remnants of degenerating nerve fibers and associated Schwann cells (Fig. 3).

V21 group. The proximal nerve stump endoneurial spaces were prominent with numerous bundles of collagen fibers of heterogenous arrangement. Endoneurial tubes were vaguely defined and irregular in shape, containing individual nerve fibers or groups of nerve fibers (Fig. 1). Schwann cells were the numerically dominant cell population, their nuclei were euchromatic, surrounded by wide belts of cytoplasm. These cells were located in the endoneurial tube next to the corresponding axon or were isolated from the fiber structure. Minifasciculi of myelinated and unmyelinated nerve fibers surrounded by a discontinuous layer of fibroblasts were observed. Myelinated nerve fibers had different axon diameters and relatively uniform myelin sheath thicknesses. External laminae were observed sporadically, mainly around individual nerve fibers (Fig. 2). In the distal nerve stump, intrafascicularly, an increased number of Schwann cell, arranged in clusters of different orientations was observed. The discontinuous endoneurial connective tissue bands demarcate irregular endoneural tubes that sporadically contain Schwann cells. (Fig. 3).

M21 group. The endoneurial connective tissue contained uniformly arranged collagen fibers. The endoneurial tubes were clearly visible, regular in shape and contained regenerating nerve fibers (Fig. 1). Schwann cells were placed inside the endoneurial tubes together with the corresponding axons. Variably thick myelinated nerve fibers withmyelin sheaths of different thicknesses were surrounded by well-structured and continuous external laminae. The myelin sheath was prominent in comparison to the Schwann cell cytoplasm and the corresponding axon. Myelinated and unmyelinated nerve fibers were predominantly organized in minifasciculi surrounded by a common external lamina and a compact layer of collagen fibers (Fig. 2). In the distal nerve stump, intrafascicularly, increased cellularity and wide

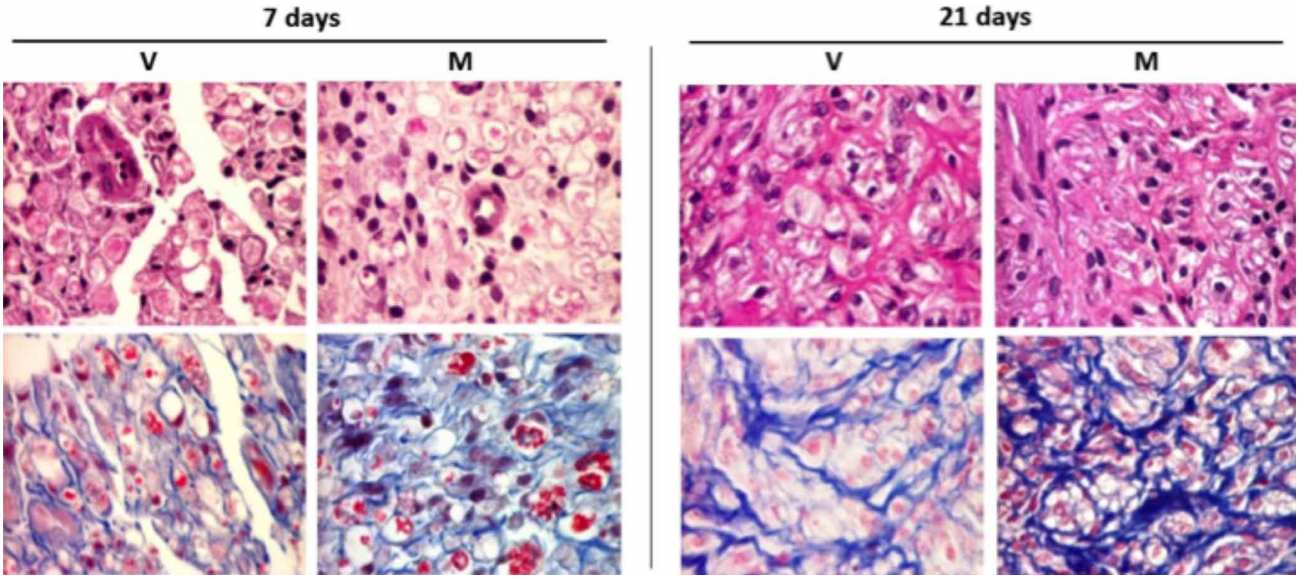

Fig. 3. Photomicrographs of the distal nerve stump in the vehicle (V) and melatonin (M) group at 7 and 21 days postoperatively, 400x (upper panels PAS, lower panels Azan).

bands of endoneurial connective tissue were noted. The uniform endoneurial connective tissue bands were composed of collagen fibers and a small number of fibroblasts. The endoneurial tubes were relatively wide and regular in shape containing numerous Schwann cells (Fig. 3).

Quantitative histological analysis. (Figs. 4 to 5 and Table I)

There is no statistically significant difference in the mean value of the endoneural tube diameter between the V7 and the M7 group (p = 0.205) nor between the V21 and the M21 group (p = 0.088).

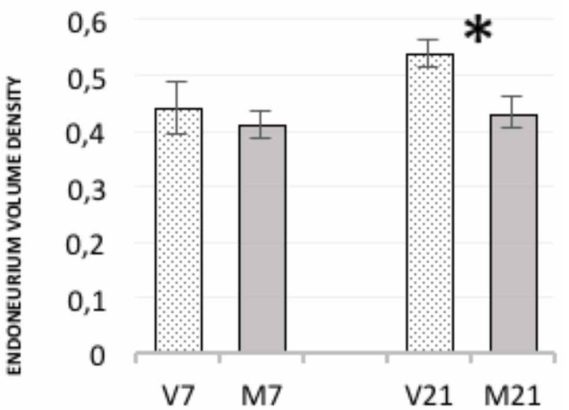

Fig. 4. Comparison of the endoneurium mean volume density (Vv) of the vehicle (V) and melatonin (M) group, at 7 days (V7 and M7) and 21 days postoperatively (V21 and M21, respectively). * p value <.0005.

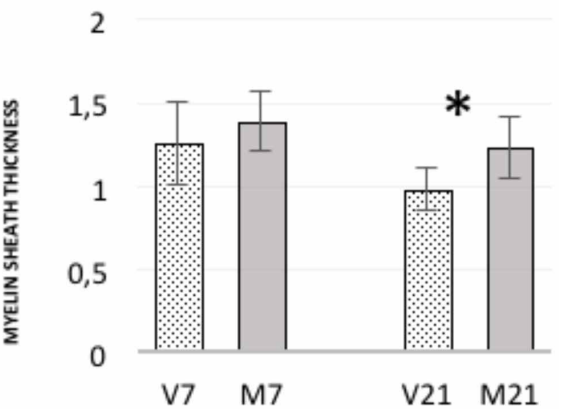

Fig. 5. Comparison of the mean myelin sheath thickness (μm) of the vehicle (V) and melatonin group (M), at 7 days (V7 and M7) and 21 days postoperatively (V21 and M21, respectively). * p value <.05.

	V		M	
	7 days	*21 days*	*7 days*	*21 days*
N	7	7	10	10
Mean±SD	7.70±0.80	15.38±2.77	9.10±1.36	19.22±2.84
95% CI	7.12-8.27	13.39-17.37	8.05-10.15	17.18-21.25
Range (min-max)	5.58-8.33	11.91-19.46	6.25-11.00	15.18-22.96
Change (%)	/	/	+ 18.18[1]	+ 24.97[1]
p value	<.0005		<.0005	

Table I. Endoneural tube diameter of the distal stump (μm) of vehiculum and melatonin group, at 7 and 21 days postoperatively.

V – vehiculum group, M – melatonin group; 1comparison with the matching vehiculum group.

DISCUSSION

Mechanical trauma is a common cause of peripheral nerve injuries that include varying degrees of morphological damage. Reconstructive microsurgery has been used in the treatment of these injuries. However, it fails to achieve full functional recovery. Thus, different additional treatment modalities have been in the focus of researchers for many years. Melatonin is a neuroprotective substance and acts as a free radical scavenger and stimulates several antioxidant enzymes (Cuzzocrea & Reiter, 2001; Tan *et al*., 2002; Rogério *et al*., 2002; Sayan *et al*., 2004; Chang *et al*., 2008). Its effects are probably also related to its direct action via MT1 receptors localized along axons and on Schwann cells (Stazi *et al*., 2021).

Nerve transection results in histological changes of intrafascicular and extrafascicular structures (Turgut *et al*., 2005; Cosovic *et al*., 2017; Kerns *et al*., 2021). To study the neuroprotective effect of melatonin as a proregenerative substance we have observed the intrafascicular structures of the rat sciatic nerve with the emphasis on degenerating and regenerating nerve fibers, changes of the myelin sheath, connective tissue of the endoneurial compartment and the endoneurial tubes.

In our study, in the vehicle treated group of rats after seven days nerve transection resulted in degenerative changes of mainly thick myelinated nerve fibers in the proximal nerve stump. In comparison to that, in the V21 group we observed predominantly regenerating myelinated and unmyelinated nerve fibers arranged in minifasciculi surrounded by a discontinuous layer of fibroblasts. The results of Fisher *et al*. (Fischer *et al*., 1985) confirm our findings regarding the presence of numerous axons of smaller diameter in the sciatic nerve after transection. We further observed moderate proliferation of Schwann cells in both vehicle groups of rats. Their external laminae in the proximal stump of the V7 group were continuous, prominent, but slightly deformed. In V21 rats the newly formed external laminae were observed sporadically around individual nerve fibers. Ether and Polock (Eather & Pollock, 1987) also found an intense Schwann cell proliferation in the sciatic nerve after axonotomy. Schwann cells produce an external lamina and limit the space within which a regenerating fiber or group of nerve fibers are located (Atkins *et al*., 2007). The research of Fisher *et al*. (Fischer *et al*., 1985) shows that 60 days after transection and nerve suture an old external lamina surrounds a newer one located in immediate vicinity to the Schwann cell. In our study, the endoneural connective tissue of the proximal stump was hypercellular in both vehicle groups. In the V7 group the endoneurium was edematous, while it was compactly arranged in the V21 group. The endoneural tubes were clearly defined in the V7 group, while they were vaguely defined and wider in the V21 group of rats. This finding is important since Atkins *et al*. (2007) have shown that intense proliferation of the endoneurial connective tissue resulted in a decrease in nerve fiber number and diameter, thus, hindering nerve tissue regeneration. Similar histological findings in the proximal nerve stump were found by Turgut *et al*. (2005) 4 weeks after sciatic nerve transection in sham pinealectomized rats and in rats with intact pineal glands, and Fischer *et al*. (1985) 30 days after rat sciatic nerve transection. The intensity of collagen accumulation after transection depends, among other things, on the type of nerve (Eather & Polock, 1987).

In the distal stump, the endoneural tubes were irregular in both groups, but their content differed: in the V7 group they were empty or contained remnants of degenerating nerve fibers and associated Schwann cells, and in the V21 group they contained sporadically Schwann cells. Our results for groups V7 and V21 are comparable to the results of Ngeow (2010) which indicate that after transection scarring and consequent reduction of the endoneurial tube diameter occur. Numerous authors emphasize the importance of the relationship between proregenerative activities of Schwann cells and connective tissue accumulation in context of nerve recovery after injury (Eather & Polock, 1987, 1988; Turgut *et al*., 2005). This is in accordance with our findings.

To assess the overall regenerative potential of the transected nerve, it is, according to our opinion, necessary to assess the state of Schwann cells and the endoneurial connective tissue as well as the condition of endoneurial tubes distal to the transection site.

In our study, we observed the protective effect of melatonin on all intrafascicular structures already in the nerve stumps of the M7. Our question was whether there was a time-dependence of the neuroprotective effects of melatonin on both nerve stumps. In the proximal nerve stump of the M7 group, we found intense Schwann cell proliferation and a condensed Schwann cell cytoplasm surrounding the regenerating axons. In case of sharp transection, axonal swelling begins as early as the fourth day after transection (Ryu *et al*., 2011), and Schwann cell proliferation begins the second day after transection, and peaks after seven days (Zhang *et al*., 2008). In our study, the endoneurial spaces were narrow, and the belt composed of collagen fibers was regularly structured and associated with the plasmalemma of Schwann cells with an external lamina between them. Both the existence of the endoneurium and the proliferative potential of Schwann cells are important factors for nerve regeneration. The proregenerative effect of melatonin was

even more pronounced in the M21 group. Thus, in the proximal nerve stump there was no significant proliferative reaction in the endoneurial connective tissue. Numerous Schwann cells together with the corresponding regenerating nerve fibers were observed inside the endoneurial tubes. Myelinated nerve fibers were numerous, especially those of smaller diameter, and the thickness of the myelin sheaths and axons were irregular. The strong neuroprotective and proregenerative effect of melatonin on disintegration of the myelin sheath and the axon was also shown by Shokouhi *et al*. (2008) in a rat model of blunt sciatic nerve injury. In our study, exogenous melatonin exerted protective and proregenerative effects on the myelin sheaths. The mean thickness of the myelin sheath was larger in group M7 (1.39 ± 0.18 μm) compared to group V7 (1.26 ± 0.24 μm), but this difference was not statistically significant ($p = 0.927$). The mean thickness of the myelin sheath in group M21 (1.23 ± 0.18 μm) was significantly larger ($p = 0.046$) than the mean diameter in group V21 (0.98 ± 0.13 μm). The degree of myelination depends, among other things, on the thickness of the axons, which according to Ngeow (2010) are of reduced diameter due to pronounced deposition of connective tissue. Also, we observed that melatonin inhibited endoneurial connective tissue proliferation as early as seven days after nerve transection. The volume density of the endoneurium in the M7 group, although not statistically significant ($p = 0.711$), was lower (0.42 ± 0.023) compared to the one in V7 group (0.44 ± 0.046), while the difference was statistically significant ($p < 0.0005$) when comparing group M21 (0.43 ± 0.03) to group V21 (0.54 ± 0.02). All this indicates that the application of large doses of exogenous melatonin after transection promotes axonal growth and suppresses collagen synthesis. In the study of Turgut *et al*. (2005) melatonin application resulted in a significant reduction of connective tissue accumulation, as well as a significantly increased share of neural elements in the nerve structure.

In the distal nerve stump of the M7 group, hyper cellularity intrafascicularly in all areas and dilated blood vessels with hypertrophic endothelium were observed. The collagen fibers of the endoneurium were relatively uniform in context of tinctorial properties forming regular endoneurial tubes of visible lumina with the remnants of nerve fibers at different stages of degeneration. In the distal nerve stump of the M21 group, increased cellularity was observed intrafascicularly, caused mainly by Schwann cell and sporadic fibroblast proliferation. Under normal conditions, cells are not numerous in the endoneurial compartment so that most collagen fibers are produced by Schwann cells (Reina *et al*. 2013). The results of our qualitative histological analysis showed a more extensive deposition of endoneurial connective tissue and narrower endoneural tubes in the distal nerve stump in group V7 vs. group M7. This was confirmed by results of our quantitative analysis showing a larger mean diameter of endoneurial tubes in the distal segment in group M7 (9.10 ± 1.36 μm) in comparison to group V7 (7.70 ± 0.80 μm), although the difference was not statistically significant ($p = 0.205$). This can be explained by the fact that in group V7 and M7 the tubes are single and correspond to a single myelinated fiber or a group of unmyelinated nerve fibers. Over time, in both groups, there were structural changes in the endoneurial connective tissue, and individual tubes confluenced into larger tubes lined by thick layers of connective tissue. The mean value of the endoneural tube diameter in the M21 group (19.22 ± 2.84 μm) was higher than in the V21 group (15.38 ± 2.27 μm), although this difference was not statistically significant ($p = 0.088$). The increase (18.18 %) in the endoneurial tube diameter after seven days and after 21 days (24.97 %) in the melatonin group compared to the vehicle group shows an inhibitory action of melatonin on scar formation.

CONCLUSION

Melatonin treatment has a time-dependent beneficial effect on the intrafascicular structures of the transected peripheral nerve through its action on nerve fiber regeneration, Schwann cell and endoneural tube preservation and scar formation. Thus, as melatonin creates better conditions for the passage of regenerating axons, it could be used as proregenerative support to reconstructive microsurgical procedures in the postoperative period.

COSOVIC, E.; KAPIC, D.; LUJINOVIC, A.; SAHINOVIC, M.; ALICELEBIC, S.; CUSTOVIC, S.; MUZIKA, V.; METOVIC, A. Efectos proregenerativos dependientes del tiempo de la melatonina exógena en el nervio ciático seccionado. *Int. J. Morphol., 41(1)*:188-194, 2023.

RESUMEN: Los procedimientos microquirúrgicos son el tratamiento de elección de las lesiones de los nervios periféricos, pero a menudo no logran una recuperación funcional completa. La melatonina tiene acciones neuroprotectoras y podría ser utilizada como un posible apoyo farmacológico proregenerativo. Por lo tanto, el objetivo de este estudio fue analizar la dependencia del tiempo del efecto neuroprotector de la melatonina sobre las estructuras fasciculares generales de ambos extremos del nervio seccionado. La sección del nervio ciático se realizó en 34 ratas Wistar macho adultas divididas en cuatro grupos: dos grupos de vehículo (N=7) tratados por vía intraperitoneal durante 7 (V7) o 21 (V21) días consecutivos con vehículo (5 % de etanol en solución Ringer) y dos grupos grupos de melatonina (N=10) a los que se les administró por vía intraperitoneal 30 mg/kg de melatonina durante 7 (M7) o 21 (M21) días consecutivos. Al final del experimento, se extir-

paron y procesaron el neuroma del muñón proximal y el fibroma del muñón distal del nervio para un análisis histológico cualitativo y cuantitativo. Las estructuras neurales intrafasciculares se conservaron mejor y el depósito de colágeno se redujo en los grupos tratados con melatonina respecto a los grupos con vehículo. La regeneración de la vaina de mielina observada a través de la medición de su espesor fue estadísticamente significativa ($p<0,05$) más pronunciada en el grupo M21 (1,23±0,18 μm) vs V21 (0,98±0,13 μm). La densidad de volumen media del endoneuro fue menor en ambos grupos tratados con melatonina en comparación con los grupos tratados con vehículo equivalente. Aunque no fue estadísticamente diferente, el diámetro del tubo endoneural fue mayor en ambos grupos de melatonina frente a los grupos de vehículo, y el efecto de la melatonina fue más pronunciado después de 21 días (aumento del 24,97 %) frente a los 7 días de tratamiento con melatonina (18,8 % de aumento). La melatonina ejerce un efecto proregenerativo dependiente del tiempo sobre las fibras nerviosas del muñón proximal y un efecto anticicatricial en ambos muñones.

PALABRAS CLAVE: Transección de nervio; Melatonina; Vaina de mielina; Endoneuro.

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