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Effect of Melatonin on Peripheral Nerve Damage Resulting from Tibial Defect in Rats

Efecto de la Melatonina sobre el Daño de los Nervios Periféricos como Resultado de un Defecto Tibial en Ratas

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YÜKSELMIS, O. & ERMIS, I. S. Effect of melatonin on peripheral nerve damage resulting from tibial defect in rats. *Int. J. Morphol., 40(4)*:1035-1042, 2022.

SUMMARY: Peripheral nerve damage (PNI) can cause demyelination, axonal degeneration and loss of motor and sensory function. Melatonin with its antioxidative effect, has been reported to reduce scar formation in nerve injury, take a role in repair process by suppressing fibroblast proliferation in the damaged area. It was aimed to investigate the effect of melatonin in the repair of peripheral nerve damage and the relationship between S100 proteins and angiogenic regulation. Wistar albino rats were divided into 3 groups. In the Defect group, 6 mm tibial bone defect using a motorized drill was created and kept immobile for 28 days . In Defect + graft group, tibial bone defect with allograft treatment was applied and kept immobile for 28 days. In Defect + graft + Melatonin group, melatonin was administered to defect + allograft group. All rats were sacrified by decapitation, skin and tibia bone were removed then fixed with 10 % neutral buffered formalin and embedded in paraffin, sections were examined under light microscopy. In the Defect+Graft group, enlargement and occlusion of the vessels with degeneration of the epineural sheath, thickening of the endoneural sheath and mild hyperplasia of schwannocytus (Schwann cells) were remarkable. In the Defect+Graft+Melatonin group, the epineural sheath was tight and regular, the axonal structures were prominent in the endoneural area. Mild S100 expression was observed in Defect+Graft group in fibers of the endoneural region with a prominent expression in schwannocytus. In Defect+Graft+Melatonin group (10mg/kg), S100 expression was moderate in areas where schwannocytus proliferated and nerve-connective tissue sheaths were reconstructed. VEGF expression was moderate in endoneural, perineural and epineural connective tissue sheaths in the Defect+Graft+Melatonin group, with negative expression in blood vessel endothelial cells, but with a positive expression in schwannocytus. We conclude that with the application of melatonin; oxidative stress decreases, schwannocytus proliferation increases, having positive influence on nerve repair with the regulation of S100 signaling and angiogenetic structuring.

KEY WORDS: Periferal nerve; Tibia Defects; S100 protein; VEGF; rat.

INTRODUCTION

Peripheral nerve lesions due to trauma can reduce quality of life with lifelong paralysis, severe sensory impairment, decreased functionality and/or pain. Significant treatment for tension-free reconnected nerve endings (Yi & Dahlin, 2010) It is the use of autologous nerve grafts. However, a secondary surgical requirement for nerve graft harvesting and loss of donor nerve function occurs (Hallgren *et al*., 2013; Faroni *et al*., 2015).

In the treatment of peripheral neuropathy, although grafts are used for traumatic nerve injury (Baradaran *et al*., 2021) and metabolic control for diabetic neuropathy (Cernea & Raz, 2021; Holmes & Hastings, 2021), reasons related to the subtype and compliance of the disease should be investigated.

Schwannocytus (Schwann cells) play an important role in physiological and pathological conditions by protecting nerve structure and functions in the peripheral nervous system and by secreting various signaling molecules and neurotrophic factors to support both axonal growth and myelination. Human peripheral nerve axons have a high regeneration capacity after trauma, which depends on the balance between Schwannocytus nerve regeneration and scar tissue.

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Melatonin (MLT), N-acetyl-5-methoxytryptamine, is a secretion synthesized by the pineal gland at night and has a strong antioxidative effect. Melatonin has been reported to be a substance that tends to reduce scar formation in nerve injury, which affects the repair process by suppressing fibroblast proliferation in the damaged area. Melatonin has many physiological roles, such as regulating blood pressure and scavenging free radicals. In particular, melatonin has been reported to have important functions in healing nerve damage due to its ability to be a potent apoptosis inhibitör (Mekaj *et al*., 2014). Melatonin (MLT), N-acetyl-5-methoxytryptamine, is a secretion synthesized by the pineal gland at night and has a strong antioxidant effect. Melatonin has been reported to be a substance that tends to reduce scar formation in nerve injury, which affects the repair process by suppressing fibroblast proliferation in the damaged area. S100 protein, an important marker in traumatic brain injury, has important effects on neural regeneration and proliferation of schwannocytus (Wang *et al*., 2015; Wu *et al*., 2015; Jiang *et al*., 2016). Vascular endothelial growth factor (VEGF) is an effective factor in the formation of neuritogenesis as well as being effective in angiogenesis and vasculogenesis. Vascular endothelial growth factor (VEGF) is an effective factor in the formation of neuritogenesis as well as being effective in angiogenesis and vasculogenesis.

As a neurotrophic and neuroprotective factor for neurons and glial cells, VEGF has been reported to stimulate proliferation of neuronal precursors (Muratori *et al*., 2018).

It was aimed to investigate the effect of melatonin in the repair of peripheral nerve damage and the relationship between S100 proteins and angiogenic regulation (VEGF).

MATERIAL AND METHOD

Ethical approval was taken from Dicle University Animal Research Committee. 30 Sprague-Dawley (250-280 g) were housed in normal cages with free access food pellet and water. Animals were assigned to three group. Defect group: a 6 mm defect was created on tibial bone and rats were kept immobilized for 28 days. Defect + graft group: a 6 mm defect was created on tibial bone, then allograft was applied and rats were kept immobilized for 28 days. Defect + graft + Melatonin(10mg/kg) group: a 6 mm defect was created on tibial bone, then allograft was applied, and rats were administered melatonine and kept immobilized for 28 days (Koparal *et al*., 2016). As part of this study, permission was obtained from the lead author, my thesis advisor, Engin Deveci, and peripheral nerve effects were studied.

At the end of study, rats were sacrificed and skin and tibia bone were excised along with nerve tissues. Tissues were stored in %10 formaldehyde solution and processed for routine parafine tissue protocol. Paraffin blocks were cut with microtome (5 μm thickness) and stained with Hematoxyline-Eosin dye.

Immunohistochemical Analysis. Immunostaining was done by method of Özevren *et al*. (2017). Antigen retrieval was done in microwave (Bosch®, 700 watt) for 3 min x 90 ºC. They were subjected to a heating process in a microwave oven at 700 watts in a citrate buffer (pH 6) solution for proteolysis. Sections were washed in 3x 5 min PBS and incubated with hydrogen peroxide (3 ml 30 % Hydrogen peroxide (H2 O2) + 27 ml methanol) for 15 min. Sections were washed in 3x5 min PBS min and blocked with Ultra V Block for 10 min. After draining, primary antibodies were directly applied to sections distinctly S100 and VEGF antibodies. Sections were incubated and left overnight at 4C. Sections were washed in 3x5 min PBS and then incubated with Biotinylated Secondary Antibody for 14 min. After washing with PBS, Streptavidin Peroxidase was applied to sections for 20 min. Sections were washed in 3x5 min PBS and DAB were applied to sections up to 15 min. Slides showing reaction was stopped in PBS. Counter staining was done with Harris's Hematoxylin for 40 s, dehydrated through ascending alcohol and cleared in xylene. Product Number: HHS32 SIGMA, Hematoxylin Solution, Harris Modified, Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103, USA. Slides were mounted with Entellan® and examined under light microscope (Zeiss, Germany).

Statistical Analysis. The data obtained in the study were expressed as arithmetic mean ± standard deviation. Statistical analyzes were made using the SPSS program. In comparison of the groups, Kruskal-Wallis test and Bonferroni corrected post-hoc test were used. $P < 0.05$ was taken as the significance level.

The data were recorded as arithmetic mean ± standard deviation with mean rank value. Statistical analysis was done using the IBM SPSS 25.0 software (IBM, Armonk, New York, US). Kruskal-Wallis test was used for multiple comparisons. Within-group comparisons, Mann-Whitney U and were used. $P < 0.05$ was used as the significance level.

RESULTS

Statistical analysis of biochemical was shown in Table I. MDA, MPO, GSH and Schwannocytus degeneration was highest in defect group, the values were decreased in defect+graft and defect+graft+melatonin groups and the decrease were statistically significant. Inflammation, vascular dilatation and S100 expression was decreased in defect+graft+melatonin compared to defect and defect+graft groups, and this decrease was statistically significant. VEGF expression was statistically decreased in melatonin treated group compared to defect group. Graphical illustration of Table I was shown in Figures 1 and 2.

Defects group: In the longitudinal section of the peripheral nerve, degeneration in the large endoneural and perineural sheath, hyperplasia in the schwannocytus, degeneration and loss in the axonal structures were observed (Fig. 3a). Defect+Graft group: Along with degeneration in the epineural sheath, blood vessels were seen as dilated and congested. Thickening of the endoneural sheath, mild hyperplasia in schwannocytus. Some axonal structures were observed (Fig. 3b). In the Defect+Graft+Melatonin group, the epineural sheath was tight and regular, the axonal structures were prominent in the endoneural area, and the nuclei of the surrounding schwannocytus were chromatin-rich and slightly hypertrophic, while a significant schawann cell proliferation was observed (Fig. 3c).

Defects group: S100 expression was increased in the fibers of the epineural sheath, in the degenerative axonal areas of the Schwannocytus nuclei (Fig. 4a). Defect+Graft group: While there was mild S100 expression in fibers in the

Table I. Biochemical (MDA, GSH and MPO) of control, spinal cord injury (SCI) and SCI+Honokiol groups.

a	Groups	n	Mean+S.D.	Kruskal-Wallis Mean Rank	H test P value	Mann-Whitney U Test ($p<0.05$)
MDA	(1) Defect	8	57.3±1.53	20.50	20.507 P=0.001	(2) (3)
	(2) Defect+Greft	8	45.43±1.30	12.50		(1)(3)
	(3) Defect+ Greft+Melatonin	8	26.87±1.10	4.50		(2) (1)
MPO	(1) Defect	8	11.18±0.59	19.63	16.245 P=0.001	(2) (3)
	(2) Defect+Greft	8	8.57±0.63	12.50		(1) (3)
	(3) Defect+ Greft+Melatonin	8	4.71±0.16	5.38		(2) (1)
GSH	(1) Defect	8	1.97±0.07	20.06	19.430 P=0.001	(2) (3)
	(2) Defect+Greft	8	1.53±0.07	12.94		(1) (3)
	(3) Defect+ Greft+Melatonin	8	0.69±0.065	4.50		(2) (1)
Schwannocytus degeneration	(1) Defect	8	3.25±0.16	18.63	16.699 P=0.001	(2) (3)
	(2) Defect+Greft	8	2.62±0.19	13.63		(1) (3)
	(3) Defect+ Greft+Melatonin	8	1.5±0.19	5.25		(2) (1)
Inflammation	(1) Defect	8	3.25±0.16	17.38	12.747 P=0.002	(3)
	(2) Defect+Greft	8	2.87±0.22	14.06		(3)
	(3) Defect+ Greft+Melatonin	8	1.87±0.22	6.06		(2) (1)
Vascular dilatation	(1) Defect	8	3.12±0.22	17.38	10.037 P=0.007	(3)
	(2) Defect+Greft	8	2.62±0.26	13.25		(3)
	(3) Defect+ Greft+Melatonin	8	1.75±0.25	6.88		(2) (1)
S100 expression	(1) Defect	8	3.12±0.22	15.75	8.038 P=0.018	(3)
	(2) Defect+Greft	8	3.00±0.26	14.63		(3)
	(3) Defect+ Greft+Melatonin	8	2.00±0.26	7.13		(2) (1)
VEGF expression	(1) Defect	8	3.25±0.25	16.75	5.370 P=0.068	(3)
	(2) Defect+Greft	8	2.5±0.26	10.75		
	(3) Defect+ Greft+Melatonin	8	2.5±0.19	10.00		(1)

endoneural region, S100 expression was prominent in some schwannocytus (Fig. 4b).

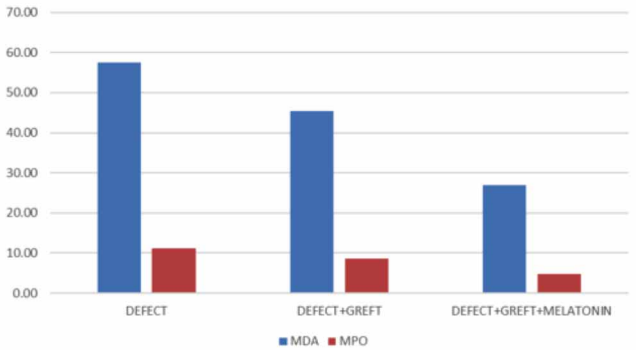

Fig. 1. Graphical illustration of MDA and MPO values.

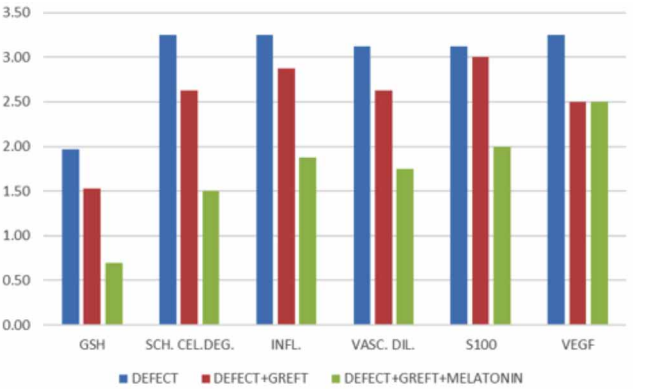

Fig. 2. Graphical illustration of GSH, Schwannocytus degeneration (sch. Cel. Deg.), inflammation (infl.), vascular dilatation (vasc. Dil.), s100 and VEGF expression

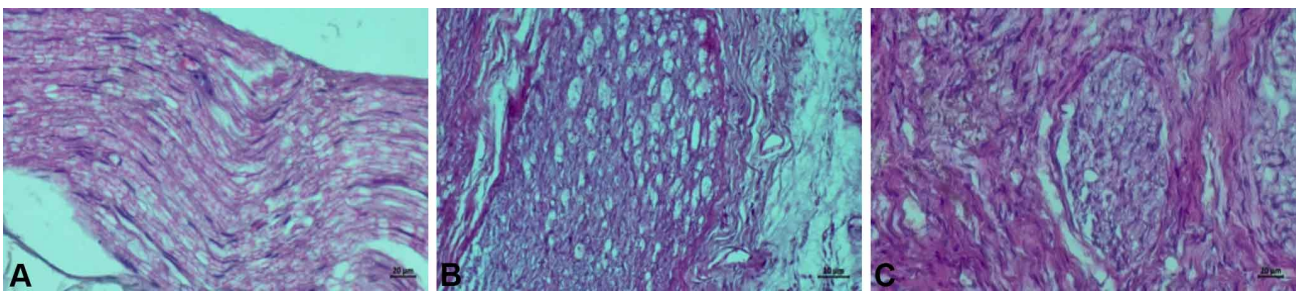

Fig. 3. Hematoxyline eosin staining. a) In defects group:, degeneration in the large endoneural/perineural sheath and acons, hyperplasic schwannocytus; b) Defect+Graft group: degenerated epineural sheath, dialted and congested blood vessels, thickening of the endoneural sheath, hyperplasic schwannocytus. c) In the Defect+Graft+Melatonin group, epineural sheath and the axonal structures were normal. Schwannocytus nuclei were chromatin-rich.

Defect+Graft+Melatonin group. It was observed that S100 expression was moderate in areas where schwannocytus proliferated and some nerve-connective tissue sheaths were reconstructed (Fig. 2c). Defects group: VEGF positive reaction was observed in Dilated blood vessel endothelial cells in the Epineural and Perineural region and in some inflammatory and degenerated axonal areas and surrounding schwannocytus (Fig. 4d).

In the immune section of the defect+graft group, VEGF expression was positive in the endoneural sheath, increased VEGF expression in capillary vessel endothelial cells, and negative expression in schwannocytus (Fig. 4e).

In the Defect+Graft+Melatonin group, VEGF expression was moderate in endoneural, perineural and epineural connective tissue sheaths, while negative expression was observed in blood vessel endothelial cells, while VEGF expression was positive in schwannocytus (Fig. 4f).

DISCUSSION

Peripheral nerve damage (PNI) can cause demyelination, axonal degeneration and loss of motor and sensory function. In the peripheral nerve damage, ischemic and inflammatory processes begin, free oxygen radicals and many toxic agents begin to accumulate around the injury site. Membrane permeability is impaired and intracellular calcium influx is stimulated. Intracellular calcium influx, in turn, activates proteolytic pathways, causing cell destruction including destruction of neurofilaments and microtubules. In the regenerative process, scar tissue develops around the regeneration area (Atik *et al*., 2011).

It has been reported that regulating axons and schwannocytus in cellular and molecular mechanisms is an effective way for neuropathy caused by peripheral nerve damage. After injury, it is important to phagocytize and assist macrophages in tearing injured axons and causing damage

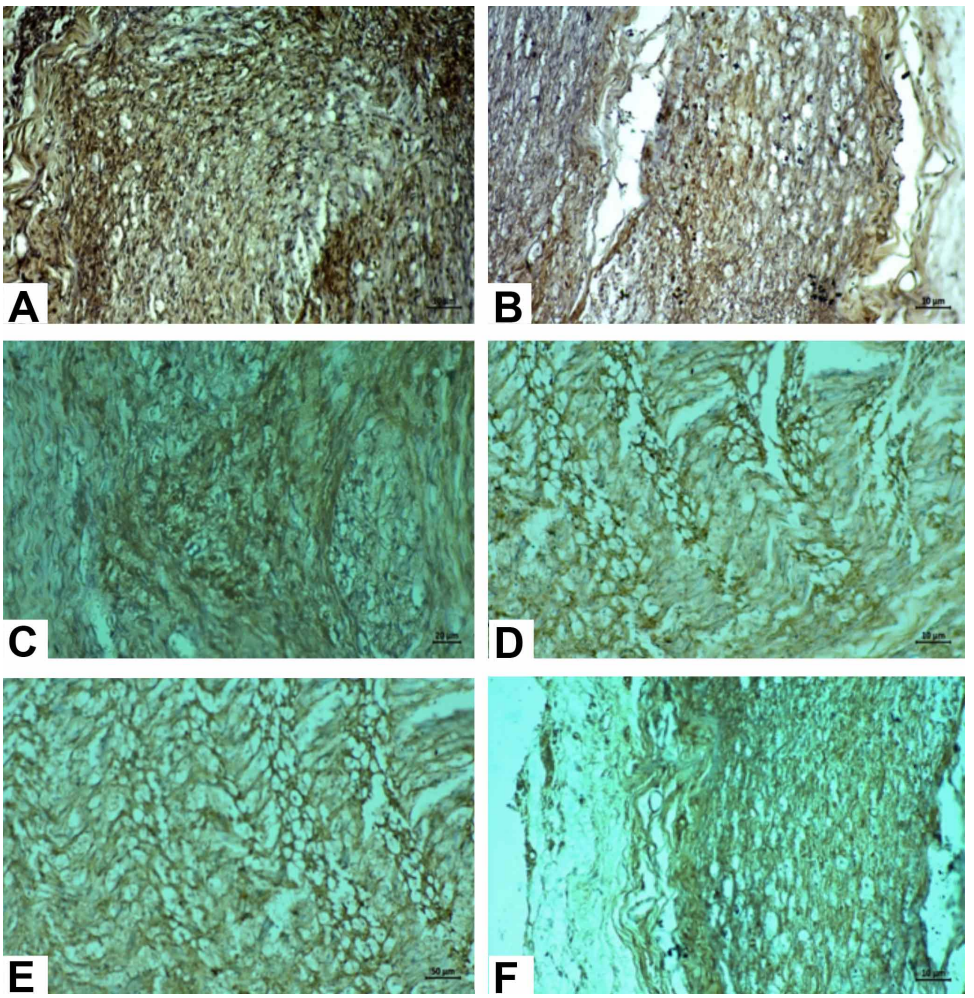

Fig. 4. Immuno staining of S100 (a-c) and VEGF (d-f).
a) Defects group: increased S100 expression in epineural sheath nad Schwannocytus.
b) Defect+Graft group: mild S100 expression in endoneural region and schwannocytus.
c) Defect+Graft+Melatonin group. Modereate S100 expression in schwannocytus and sheaths.
d) Defects group: positive VEGF expression in endothelial cells, axons and Epineural and Perineural region.
e) In defect+graft group, increased VEGF expression in the endoneural sheath and endothelial cells.
f) In the Defect+Graft+ Melatonin group, moderate VEGF expression in endoneural, perineural and epineural connective tissue sheaths.

to the distal region, called Wallerian degeneration, and subsequently compartmentalizing and reassembling this damage by schwannocytus (Nazareth *et al*., 2021).

Zhang *et al*. (2002) tried to create a 4 cm defect in rabbit tibial nerve and then repair it with autologous vein graft, autologous vein graft containing transplanted schwannocytus and conventional vein graft. They revealed axon regeneration in vessels with schwannocytus (Zhang *et al*., 2002). The obvious treatment (Yi & Dahlin, 2010) for tension-free reconnected nerve endings is the use of autologous nerve grafts. However, collection of nerve grafts results in a secondary surgical requirement and loss of donor nerve function (Hallgren *et al*., 2013; Faroni *et al*., 2015).

In the defect treated group, degeneration in the endoneural and perineural sheath, hyperplasia in schwannocytus, significant degeneration in the axonal structures, perineural sheath, hyperplasia in the endothelial cells of the vessels and dilatation in the wall structure were observed. In the Defect+Graft group, degeneration of the epineural sheath, thickening of the endoneural sheath, and mild hyperplasia of schwannocytus were observed. In the Defect+Graft+Melatonin group, proliferation in the epineural and endoneural areas, in the axonal structures, and in the schwannocytus. Restructuring in the peripheral nerve section was observed to be evident in some areas.

S100 expression levels serve as markers for the proliferation of schwannocytus in sciatic nerve regeneration studies (Wang *et al*., 2015; Wu *et al*., 2015; Jiang *et al*., 2016). Wu *et al*. (2015) used S100 protein levels in schwannocytus to indicate the extent and duration of third-degree hindpaw burn injury affecting the sciatic nerve.

Wang *et al*. (2015) reported that ginsenoside R greatly increased S100 expression in schwannocytus to promote rat sciatic nerve regeneration, and this is due to the extracellular signaling regulated kinase 1/2 and c-Jun N-terminal kinase 1/2 signaling pathways.

PNI in the acute phase can lead to the development of differentiation of schwannocytus. With the redifferentiation of schwannocytus over a period of time, the formation of new myelin sheath and axonal wrapping becomes necessary for functional neural recovery.

In the early period (2-4 weeks) after PNI, thickening of the nerve occurs due to the formation of new myelin. S100 expression of schwannocytus is used to show the amount of myelination, and S100 immunoreactivity is related to the amount of myelin in schwannocytus (Mata *et al*., 1990). After nerve injury, when the amount of schwannocytus and myelin production and nerve thickness reach a maximum, S100 immunoreactivity is also at its maximum in the second week; After 4 weeks, myelin degeneration occurs and S100 expression decreases (Liu *et al*., 2016).

It has been reported that melatonin has neuroprotective and antioxidative effects and can reduce oxidative stress by stimulating antioxidant enzymes such as superoxide dismutase (SOD), catalase (Ct), peroxidase and ascorbate peroxidase (Tan *et al*., 2007). The protective effect of MLT was found to significantly increase MDA concentration after I/R in rats that underwent 2 hours of sciatic nerve ischemia followed by 3 hours of reperfusion, while SOD levels decreased. MLT treatment has been shown to reduce the I/R-induced increase in MDA and increase the decrease in SOD levels (Sayan *et al*., 2004).

In our study, a significant increase was observed in the MDA value in the defect group, while a decrease was observed in the MPO and GSH values. Although theincrease in the MDA value in the defect+graft applied group decreased partially, the decrease in the MPO and GSH values continued. A significant decrease in MDA value and an increase in MPO and GSH values were observed in the group administered melatonin. It was considered as an important sign in regulating oxidative stress.

Chang *et al*. (2014) determined its effect on promoting Schwannocytus proliferation and improving nerve regeneration after PNI. Their results showed that the therapeutic use of MLT could be a promising strategy to counteract PNI-induced neuronal disability. It has been defined that MLT has positive effects on axon number and myelin sheath thickness by preventing collagen deposition and neuroma formation after traumatic events in peripheral nerves (Aktaş *et al*., 2009). Degeneration of the endoneural and perineural sheath, hyperplasia of schwannocytus, and degeneration of axonal structures were observed in the defect group. In the Defect+Graft group, enlargement and occlusion of the vessels with degeneration of the epineural sheath. Thickening of the endoneural sheath and mild hyperplasia of schwannocytus were remarkable.

In the group administered melatonin, the epineural sheath was tight and regular, the asonal structures were prominent in the endoneural region, the nuclei of the surrounding schwannocytus were chromatin-rich and slightly hypertrophic, while a significant Schwannocytus proliferation was observed. In the group administered melatonin, the epineural sheath was tight and regular, the asonal structures were prominent in the endoneural region, the nuclei of the surrounding schwannocytus were chromatin-rich and slightly hypertrophic, while a significant schawann cell proliferation was observed.

Wu *et al*. (2015) S100 protein levels in schwannocytus to indicate the extent and duration of third-degree burn injury affecting the sciatic nerve, Wang *et al*. (2015) found that ginsenoside Re greatly increased S100 expression in schwannocytus to promote rat sciatic nerve regeneration, and this was due to the extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase 1/2 signaling pathways.

In another study, it was reported that daily intragastric curcumin administration for 1 week induced S100 immunoreactivity in schwannocytus, had a significant effect on schwannocytus, myelin structure and functional improvement of the sciatic nerve in 8 weeks (Liu *et al*., 2016). In the defect group, VEGF positive reaction was observed in Dilated blood vessel endothelial cells in Epineural and Perineural regions, some inflammatory and degenerated axonal areas and surrounding schwannocytus. In the defect group, S100 expression was increased in the epineural sheath, degenerative axonal areas of the Schwannocytus nucleus, and mild S100 expression was observed in the fibers in the Endoneural region of the Defect + Graft gruta, while S100 expression was prominent in some schwannocytus. It was observed that S100 expression was moderate in areas where schwannocytus proliferated and some nerve-connective tissue sheaths were restructured with melatonin application.

VEGF plays a role in central nervous system and peripheral nervous system development.

The vascular and nervous systems act by common molecular signaling pathways during development and regeneration. In the early stages after peripheral nerve crush injury, VEGF mRNA expression was significantly up-regulated, while a strong down-regulation occurred in the degenerating nerve, suggesting a possible role during the regenerative process (Muratori *et al*., 2018).

Endoneural sheath, increase in VEGF expression in capillary vessel endothelial cells, negative expression in schwannocytus in the Defect+Graft group,In melatonin application, VEGF expression was moderate in endoneural, perineural and epineural connective tissue sheaths, while negative expression was observed in blood vessel endothelial cells, while VEGF expression was positive in schwannocytus.

CONCLUSIONS

It has been observed that oxidative stress mechanisms such as connective tissue sheath degeneration, Schwannocytus degeneration and deterioration in endothelial function have changed in peripheral nerves damaged as a result of tibial defect.

It has been concluded that graft application is not sufficient for the repair of nerve damage, but with the application of melatonin, oxidative stress decreases, Schwannocytus proliferation increases, and important effects may occur in nerve repair with the regulation of S100 signaling and angiogenetic structuring.

YÜKSELMIS O. & ERMIS, I. S. Efecto de la melatonina sobre el daño de los nervios periféricos como resultado de un defecto tibial en ratas. *Int. J. Morphol., 40(4)*:1035-1042, 2022.

RESUMEN: El daño a los nervios periféricos puede causar desmielinización, degeneración axonal y pérdida de la función motora y sensorial. Se ha informado que la melatonina, con su efecto antioxidante, reduce la formación de cicatrices en lesiones nerviosas y desempeña un papel en el proceso de reparación al suprimir la proliferación de fibroblastos en el área dañada. El objetivo de este trabajo fue investigar el efecto de la melatonina en la reparación del daño de los nervios periféricos y la relación entre las proteínas S100 y la regulación angiogénica. Ratas albinas Wistar se dividieron en 3 grupos. En el grupo Defecto, se creó un defecto óseo tibial de 6 mm con un taladro motorizado y se mantuvo inmóvil durante 28 días. En el grupo Defecto + injerto, se aplicó tratamiento de defecto óseo tibial con aloinjerto y se mantuvo inmóvil durante 28 días. En el grupo Defecto + injerto + Melatonina, se administró melatonina al grupo defecto + aloinjerto. Todas las ratas fueron sacrificadas por decapitación, se extrajo la piel y el hueso de la tibia y luego se fijaron con formalina tamponada neutra al 10 % y se incluyeron en parafina, las secciones se examinaron bajo microscopía óptica. En el grupo Defecto+Injerto, fueron notables el agrandamiento y la oclusión de los vasos con degeneración de la vaina epineural, engrosamiento de la vaina endoneural e hiperplasia leve de los schwannocitos (neurolemnocitos). En el grupo Defecto+Injerto+Melatonina, la vaina epineural era estrecha y regular, las estructuras axonales eran prominentes en el área endoneural. Se observó expresión leve de S100 en el grupo Defecto+Injerto en fibras de la región endoneural con una expresión prominente en los schwannocitos. En el grupo Defecto+Injerto+Melatonina, la expresión de S100 fue moderada en áreas donde proliferaron los schwannocitos y se reconstruyeron las vainas de tejido conectivo nervioso. La expresión de VEGF fue moderada en vainas de tejido conectivo endoneural, perineural y epineural en el grupo Defecto+Injerto+Melatonina, con expresión negativa en células endoteliales de vasos sanguíneos, pero con expresión positiva en schwannocitos. Concluimos que con la aplicación de melatonina; disminuye el estrés oxidativo, aumenta la proliferación de schwannocitos, influyendo positivamente en la reparación nerviosa con la regulación de la señalización S100 y la estructuración angiogenética.

PALABRAS CLAVE: Nevro periférico; Defectos de tibia; Proteína S100; VEGF; Rata.

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