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Possible promoting effects of melatonin, leptin and alcar on regeneration of the sciatic nerve

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Abstract

Peripheral nerve injury is a widespread and disabling condition that can impair the individual's daily life. Studies involving medications that may positively affect peripheral nerve regeneration are rare. The aim of this study was to investigate new treatments after peripheral nerve injury using various neuroprotectants, melatonin, alcar and leptin, in the regenerative process in an experimental rat model. Wistar albino rats were randomly divided into eight groups containing equal number of animals. Intraperitoneal injection of melatonin (50 mg/kg, for 21 days), leptin (1 mg/kg, for 21 days) and acetyl-L-carnitine (50 mg/kg, for six weeks) was performed postoperatively. Histological and electromyographical assessments of the regenerated nerves were performed 12 weeks after surgery. Stereological analysis was performed to estimate myelinated and unmyelinated axon numbers, surface area, myelin thickness and the myelin thickness/axon diameter ratio for each group. The results showed that only alcar has a beneficial effect on the regeneration of unmyelinated axons. Neither melatonin and leptin nor alcar were observed to have any therapeutic effect on the regeneration of myelinated axons. Alcar therapy has a positive effect on the regeneration of unmyelinated fiber in the sciatic nerve. However, the same effect was not observed in myelinated nerve fibers after intraperitoneal application of melatonin and leptin.

1. Introduction

Varying degrees of nerve damage, including tension, crush or discontinuation, may occur as a result of events such as falls or industrial or motor vehicle accidents. Although nerve injuries do not usually lead to life-threating conditions, they may still cause a social burden in terms of economic costs (Rosberg et al., 2013). Although there has been considerable research into peripheral nerves, the treatment of peripheral nerve injuries is still controversial (Campbell, 2008). Major efforts have therefore been dedicated to resolving this issue. The degree of damage, at the axonal level, is the most important factor determining the speed and duration of recovery following nerve injury. Although, the recovery rate in damaged nerves may vary depending on the pathophysiological situation in the injured area, fully functional recovery generally occurs so long as axonal integrity is preserved (Allodi et al., 2012; Lundborg and Richard, 2003; Robinson, 2000). Studies regarding the effects of neuroprotective agents that potentially increase axonal regeneration following peripheral nerve damage, especially if axonal integrity cannot be preserved, are therefore needed. A number of studies have investigated the neuroprotective effects of melatonin (Aygun et al., 2012; Kaplan et al., 2011; Leon et al., 2005). This has a potential neuroprotective effect in many conditions affecting the nervous system due to its lipophilic-hydrophilic and free radical scavenger properties. It can therefore protect the nucleus, cell

membranes and organelles in particular against free radical damage (Aygun et al., 2012). Melatonin has also been reported to reduce neuronal death after peripheral axotomy. This has been attributed to the preservation of motor neurons and reduction of cell loss in the dorsal horn (Odaci and Kaplan, 2009). In addition, exogenous melatonin administration after nerve injury has a positive effect on myelin sheath thickness and axon numbers by reducing collagen deposition and preventing neuroma (Turgut and Kaplan, 2011). Melatonin thus exhibits neuroprotective effects by increasing the regeneration process after peripheral nerve injuries and due to its antioxidant properties (Odaci and Kaplan, 2009). In the literature, numerous studies have investigated the neuroprotective and neurogenetic effects of leptin (Avraham et al., 2011; Folch et al., 2012; Perez-Gonzalez et al., 2011). Exogenous leptin administration reduces neuronal damage following ischemia and stroke. It has also been reported to exhibit neurogenetic and neuroprotective effects by inducing an increase in the number of neurons and glia cells by affecting neural stem cell development (Avraham et al., 2011; Folch et al., 2012; Perez-Gonzalez et al., 2011). Alcar administration increases axonal regeneration after nerve injury and produces a significant improvement in motor functions. It significantly reduces nerve loss in the dorsal root ganglia in a dose-dependent manner (Flatters et al., 2006). Alcar is thought to exhibit all its neuroprotective effects by increasing intracellular neurotrophic pathways or cholinergic neurotransmission (Flatters et al., 2006; Ori et al., 2002). This study evaluated the possible effects of some neuroprotective agents on sciatic nerve regenera- tion using stereological and electrophysiological techniques and electron microscopy.

2. Materials and methods

Histological follow-up was performed at the Histology and Embryology Department of Ondokuz Mayıs University, Turkey, and electron microscopic analysis in the laboratory of the Cavalieri Ottolenghi Neuroscience Institute, the University of Turin, Italy. The Animal Experiments Committee and the Ethics Committee of Ondokuz Mayıs University approved the study. All available precautions were taken to minimize pain, suffering and the number of animals used. At the end of the experiment, rats were sacrificed through an overdose of anesthetic. Forty-eight female Wistar albino rats (150–200 g) were divided into eight groups: a pure control group (PG) which didn't undergo any special protocol, a control gap group (CG), a melatonin group (MG) that was given only melatonin, a melatonin with gap group (MGG), a leptin group (LG), a leptin with gap group (LGG), an alcar group (AG), and an alcar with gap group (AGG). Rats received a standard diet over the 12-weeks study period. In the gap groups, following resection of the sciatic nerve, a 5 mm gap was opened between the distal and proximal parts of the nerve and the segment was rotated for 180 _ was replaced by a fibrin coated tubular scaffold. At this point; gap style is suitable model for examining whether there is a possible regeneration. Melatonin (Sigma-Aldrich) at 50 mg/kg (Keskin et al., 2015) was daily injected intraperitoneally in the melatonin groups for 21 days. During the same period, 1 mg/kg leptin (Maniscalco and Rinaman, 2014) (Sigma-Aldrich) was administered intraperitone- ally to the leptin groups. Additionally, 50 mg/kg alcar (McKay-Hart et al., 2002) (Sigma-Aldrich) was administered intraperitoneally to the alcar groups in the postoperative period. The same protocols of injection have been performed on rats of groups with sciatic nerve not transected. Histological and electromyographical assessments of the regenerated nerves were performed 12 weeks after surgery. On the 12th week postoperatively, electrophysiological analysis was performed on all rats before sacrifice. Compound muscle action

potentials of the gastrocnemius muscle were measured. The time between the beginning of stimulation and the start of deflection (latency) and peak-to-peak amplitude (p-p amplitude) of the existing potential were measured on the action potential curves. Electrophysiological analyses were performed in the Physiology Department laboratory of the Ondokuz Mayıs Univer- sity Faculty of Medicine. Electromyography (EMG) tests were performed using a 4SP PowerLab (AD Instruments, Sydney, Australia) device and Scope (ver. 3.7.2, AD Instruments) software. After the electrophysiological measurements, nerve samples were processed for resin embedding and the distal part was trans- versally sectioned using an ultra-microtome (Leica Ultracut UCT, Leica Microsystems GmbH, Germany). Sections of 70-nm were used, for the electron microscopic (JEM-1010, JEOL, Tokyo, Japan) analysis, whereas sections of 2.5 mm thickness were used for light microscopic examination. The number of unmyelinated axons (in the electron microscope images) and the number of myelinated axons, axonal and myelin sheath thickness measurements (in the light microscope images) were evaluated using stereological method which is called 2D fractionator (Stereoinvestigator 9.0. Micro Brield Field, Colchester, USA) (Kaplan et al., 2012).

2.1. Stereological analyses

The basic steps of the fractionator technique that is used in the estimation of the total myelinated axon number in peripheral nerve can be summarized as this: The nerve tissue samples belonging to each subject, following the proper histological tissue processing, should be prepared as blocks in the mounting medium appropriate for the examination method. The important factor in the block preparation is to execute the tissue processing with care in accordance with the properties of the study, size of the tissue samples and characteristics of the tissue. After the preparation of blocks, sectioning can be performed. Because, the peripheral nerve fascicle displays the same features regarding the axonal structure, one section from each block should be enough. Due to the fractionator logic, the systematic sampling will be done on every section optically. For this end, after the sections are properly stained, the number of the parameters of interest (myelinated axon number etc.) can be determined under the microscope. When the counting of the concerning parameters, such as the number of the myelinated axons, are performed, the resulting value depends on their physical existence. Namely, all the myelinated axons in the tissue have an equal chance to be sampled and it is impossible to reach a counting value greater than the number of axons that are present. Thus, the important point in the fractionator method is whether the parameter to be counted is visible. For this reason, it is crucial to render the particles of interest visible by staining processes done on sections, to prevent the result from be affected by the errors (Ross and Pawlina, 2011). When the myelinated axons on the sections are counted under microscope, it requires that the counting areas are as stated above, optically fractionated in accordance with systematic sampling method. If not, the counted particle number would be higher. This type of sampling is called area fractionators (Gundersen, 1986). Firstly, the boundaries of the area of interest are determined and it is decided how the step intervals will be in moving on this area. Area fractioning (in which the ratio of the steps that is to be sampled for counting is learned) is performed on the section by moving on the x and y axes and the myelinated axons are counted by using a counting frame step by step in each area. The size of the counting frame and the step size that limit the movement on x and y axes should be determined optimally during the pilot study by taking the properties like diameter of the nerve tissue, fascicle number etc. into account. The ratio of the counting frame area to step size gives us the area sampling ratio. As a

result, by multiplying the total number of myelinated axons counted in a step by inverse of all the used fractionator steps, the total number of myelinated axons in the nerve fascicle is gained (Fig. 1). When it is considered that the estimation is done on only one sample, it is seen that it is very probable that the total myelinated axon number belonging to the peripheral nerve sample of interest is higher than the real value. However, by increasing the number of subjects in each group and consequently repeating the estimation of the total myelinated axon number in the concerning nerve tissue with different biological variables, the calculation will be closer to the real value. That is because that the fractionator method depends on the systematic sampling basis which enables us to reach to values closest to the real in short time (Onger et al., 2014). Additionally; the same stereological workstation was also used for stereological analyses of myelin thickness and axon cross-sectional area. A two-dimensional isotropic uniform random nucleator method (Gundersen, 1986) was used for estimation of cross-sectional axon area and the thickness of myelin sheet.

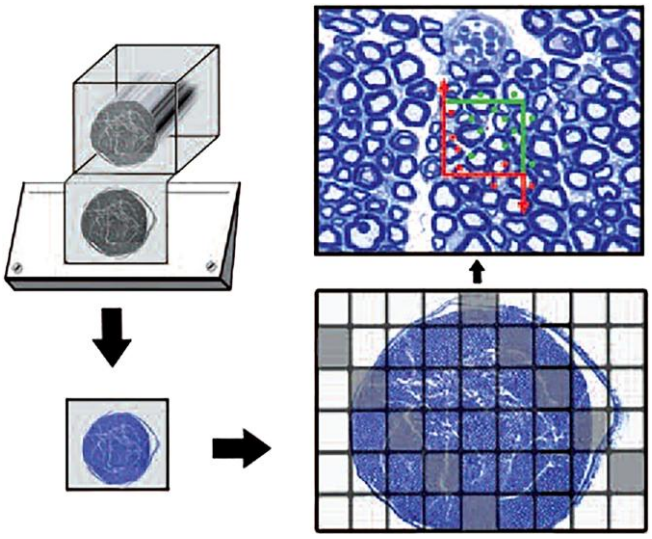

Fig. 1. The schematic summary of the estimation of the axon number in the peripheral nerve by using fractionator method. After the section that is taken from the concerning tissue block is stained with the proper dye, certain areas are chosen under the microscope in accordance with systematic sampling method (dark grey). In every chosen area, the concerning parameters are counted by using an unbiased counting frame (Onger et al., 2014).

2.2. Statistical analysis

Statistical analysis of the data was performed using SPSS 21.0 for Mac (IBM Corporation) software. One-way ANOVA (Tukey post-hoc test) was used for group comparisons, and significance was set at 0.05 (p).

3. 3. Results

3.1. Melatonin groups

3.1.1. Myelinated and unmyelinated axon numbers

In terms of myelinated and unmyelinated axon numbers, no significant difference was determined between PG and MG, or CG and MGG ($p > 0.05$). In contrast, both myelinated and unmyelinated axon numbers were significantly different between PG and MGG, CG and MG, and MG and MGG ($p < 0.01$) (Figs. 2, 7 and 8). 3.1.2. Myelinated axon area, myelin thickness and myelin thickness/ axon diameter ratio In terms of myelinated axon area (Fig. 3) and myelin sheath thickness (Fig. 4),

there was highly significant differences between PG and MG, PG and MGG, CG and MG, and MG and MGG ($p < 0.01$). However, there was no difference between CG and MGG ($p > 0.05$). Concerning the myelin thickness/axon diameter ratio; highly statistically significant difference was observed between PG and MG, PG and MGG ($p < 0.01$) (Fig. 4). Also, there was no significant difference between CG and MG, CG and MGG, MG and MGG ($p > 0.05$). 3.1.3. Amplitude and latency Comparison of amplitude values revealed no significant differences between PG and MG, or MG and MGG ($p > 0.05$), MG and MGG values were as much as 7-fold higher than those of CG (Fig. 5). In terms of latency, a highly significant difference was observed between PG and MG, and between PG and MGG ($p<0.01$) (Fig. 6). No significant difference was determined between CG and MG, CG and MGG, or MG and MGG ($p > 0.05$).

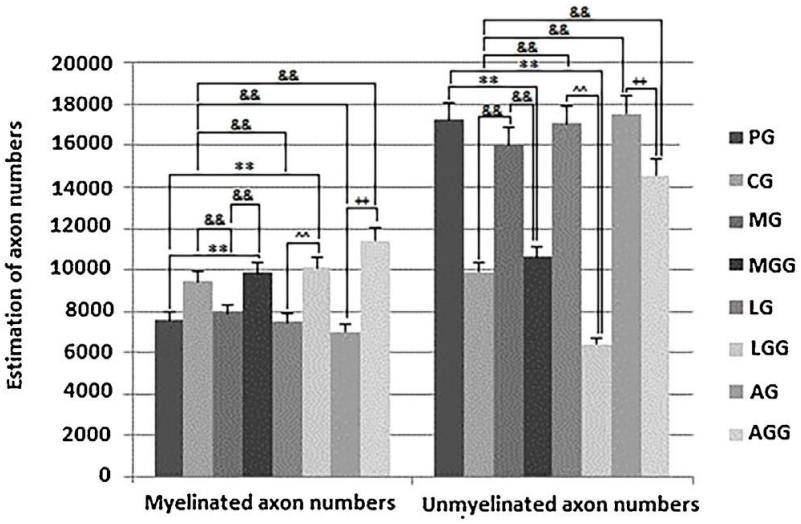

Fig. 2. Myelinated and unmyelinated axon numbers in the control and gap groups. Both myelinated and unmyelinated axon numbers were significantly different between PG and MGG, CG and MG, and MG and MGG ($p < 0.01$). Morever; highly significant differences were observed between PG and LGG, CG and LG, and LG and LGG ($p < 0.01$). Additionally, significant differences were observed between CG and AG, CG and AGG, and AG and AGG ($p < 0.01$). (**), (&&), (^^), (++) show the statistically highly significant differences between groups ($p < 0.01$).

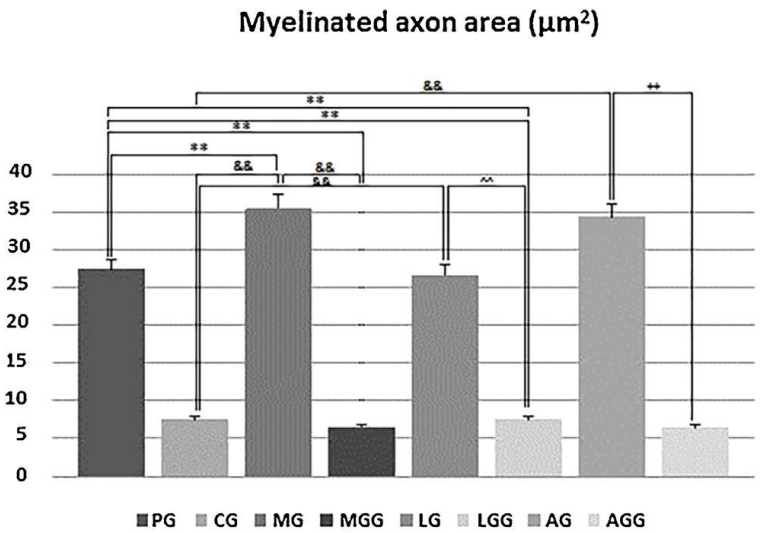

Fig. 3. Myelinated axon areas belong to the control and gap groups. There was highly significant differences between PG and MG, PG and MGG, CG and MG, and MG and MGG ($p < 0.01$). Furthermore; it was observed highly significant differences between PG and LGG, CG and LG, and LG and LGG ($p < 0.01$). Additionally; there was highly significant differences between the groups of PG and AGG, CG and AG, AG and AGG ($p < 0.01$) comparing to difference between CG and AGG. (**), (&&), (^^), (++) show the statistically highly significant differences between groups ($p < 0.01$).

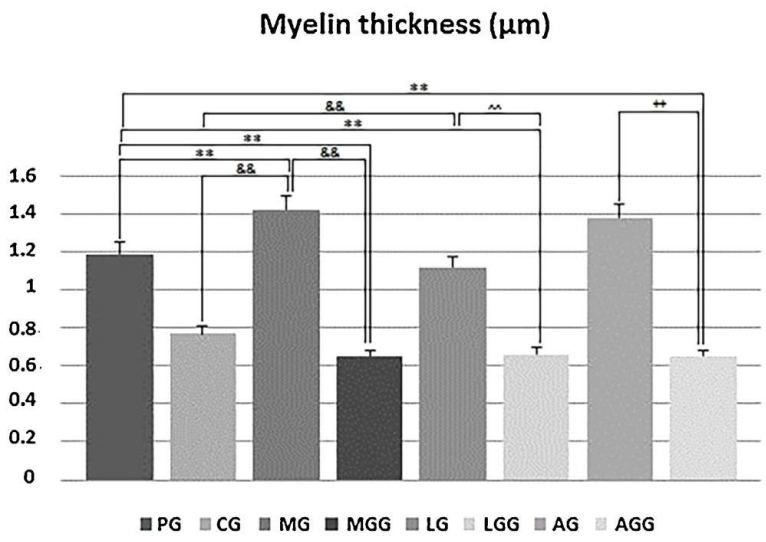

Fig. 4. Myelin thicknesses belong to the control and gap groups. There was highly significant differences between PG and MG, PG and MGG, CG and MG, and MG and MGG ($p < 0.01$). Furthermore; it was observed highly significant differences between PG and LGG, CG and LG, and LG and LGG ($p < 0.01$). Additionally; there was highly significant differences between the groups of PG and AGG, CG and AG, AG and AGG ($p < 0.01$) comparing to difference between CG and AGG. (**), (&&), (^^), (++) show the statistically highly significant differences between groups ($p < 0.01$).

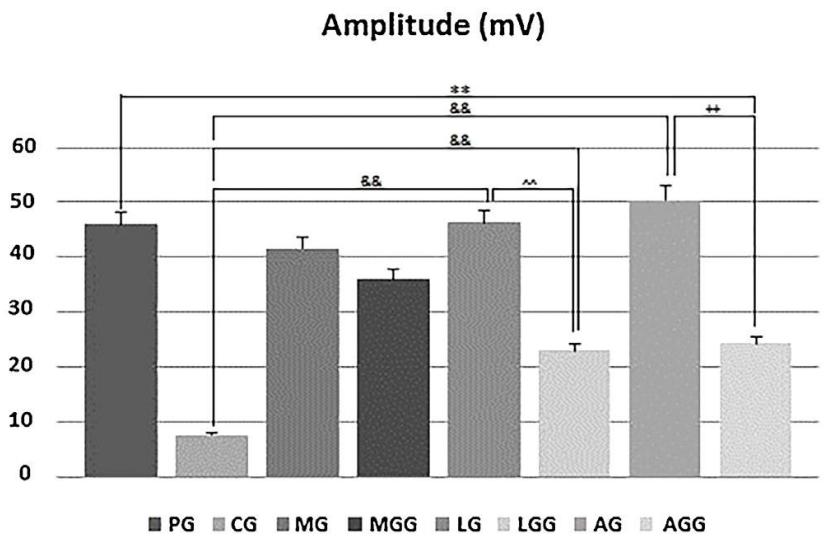

Fig. 5. Amplitude values in the control and gap groups. PG and LGG, CG and LG, CG and LGG, LG and LGG showed highly significant differences ($p < 0.01$). Morever; there was a highly significant differences between PG and AGG, CG and AG, AG and AGG ($p < 0.01$). (**), (&&), (^^), (++) show the statistically highly significant differences between groups ($p < 0.01$).

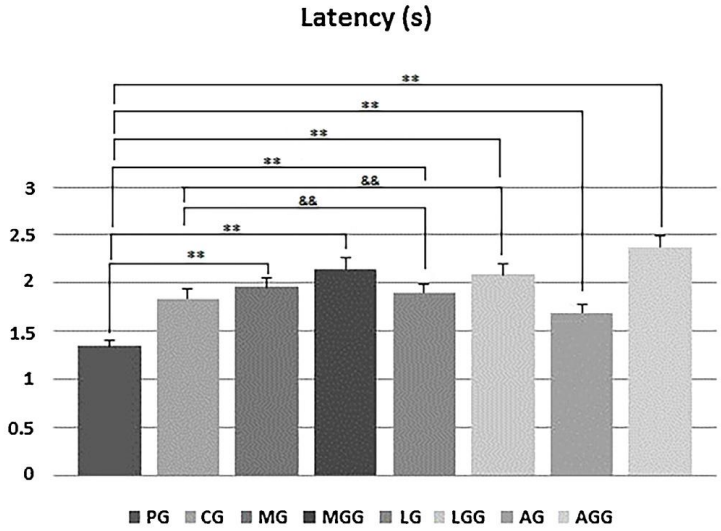

Fig. 6. Latency values in the control and gap groups. In terms of latency, a highly significant difference was observed between PG and MG, and between PG and MGG ($p < 0.01$). Morever; LG and LGG exhibited higher latency values compared to the CG, although a significant difference was only attained in the PG and LG, PG and LGG ($p < 0.01$). Additionally; there was a highly significant differences between PG and AGG, PG and AG ($p < 0.01$). (**), (&&) show the statistically highly significant differences between groups ($p < 0.01$).

3.2. Leptin groups

3.2.1. Myelinated and unmyelinated axon numbers

We determined no differences between PG and LG ($p > 0.05$) in terms of numbers of myelinated and unmyelinated axons, however, highly significant differences were observed between PG and LGG, CG and LG, and LG and LGG ($p < 0.01$) (Figs. 2, 7 and 8). 3.2.2. Myelinated axon area, myelin thickness and the myelin thickness/axon diameter ratio Differences in levels of myelinated axon area (Fig. 3) and myelin sheath thickness (Fig. 4) were observed between PG and LGG, CG and LG, and LG and LG ($p < 0.01$). Myelin thickness/axon diameter ratio was significantly different between PG and LG, PG and LGG, LG and LGG, but no significant differences were exhibited by the PG and LG, CG and LGG ($p > 0.05$). 3.2.3. Amplitude and latency PG and LGG, CG and LG, CG and LGG, LG and LGG showed highly significant differences, while the PG and LG didn't show any statistically differences for amplitude values (Fig. 5). LG and LGG exhibited higher latency values compared to the CG, although a significant difference was only attained in the PG and LG, PG and LGG ($p < 0.01$) (Figs. 5 and 6).

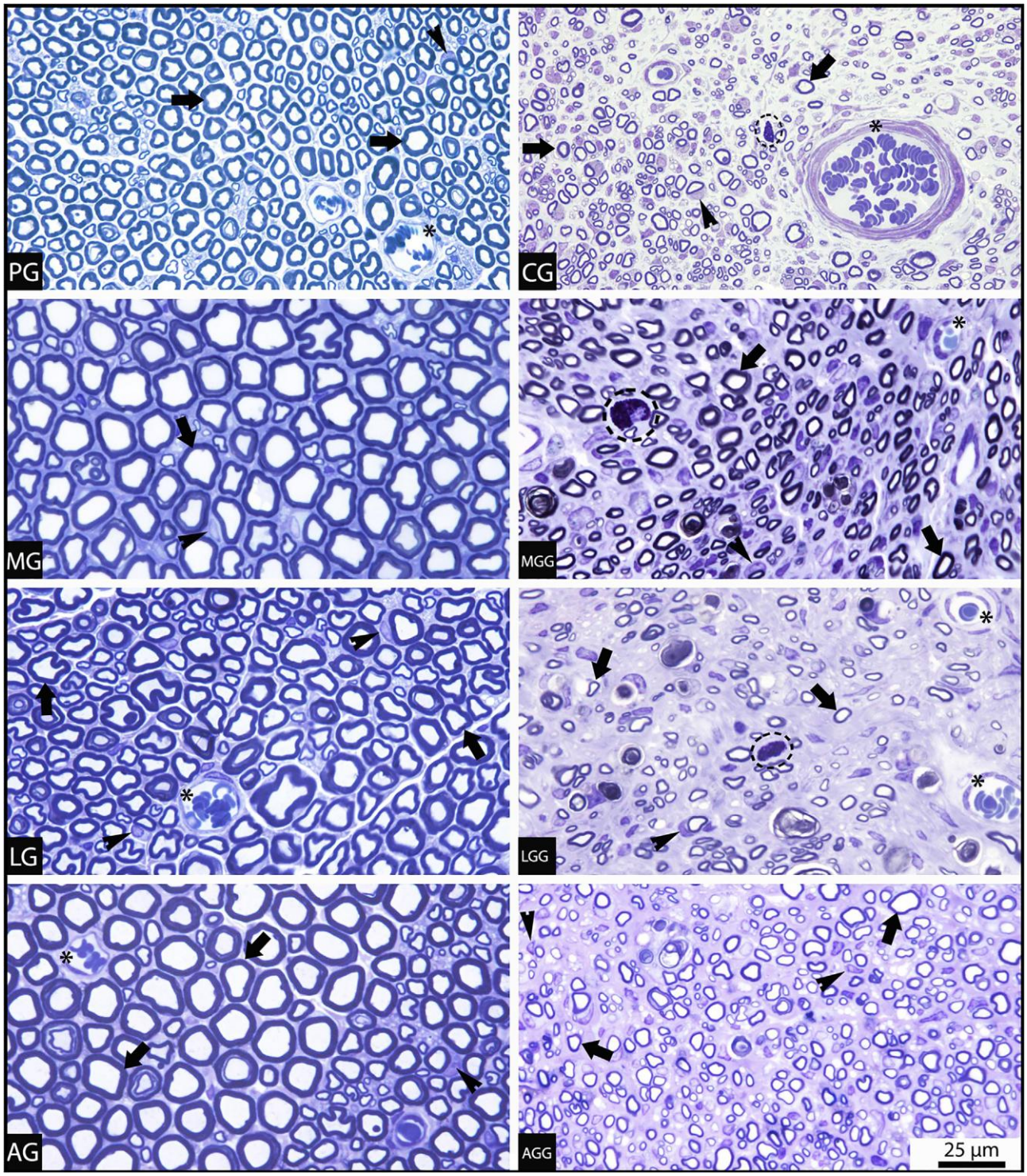

Fig. 7. Histopathological light microscopic images of the gap and control groups (thick arrows indicate myelinated axons, arrowheads indicate the nucleus of Schwann cells, stars show the blood vessels in the connective tissue, and dashed-line circles point out macrophages).

3.3. Alcar groups

3.3.1. Myelinated and unmyelinated axon numbers

One-way ANOVA revealed no differences between PG and AG in terms of numbers of myelinated and unmyelinated axons, although significant differences were observed between CG and AG, CG and AGG, and AG and AGG ($p < 0.01$) (Figs. 2, 7 and 8).

3.3.2. Myelinated axon area, myelin thickness and the myelin thickness/axon diameter ratio

Alcar intake significantly elevated axon area and myelin sheath thickness. There was highly significant differences between the groups of PG and AGG, CG and AG, AG and AGG ($p < 0.01$) comparing to difference between CG and AGG. For myelin thickness/axon diameter ratio, significant differences between groups were observed, although a slight trend of mean difference was observed between PG and AG, CG and AGG ($p > 0.05$).

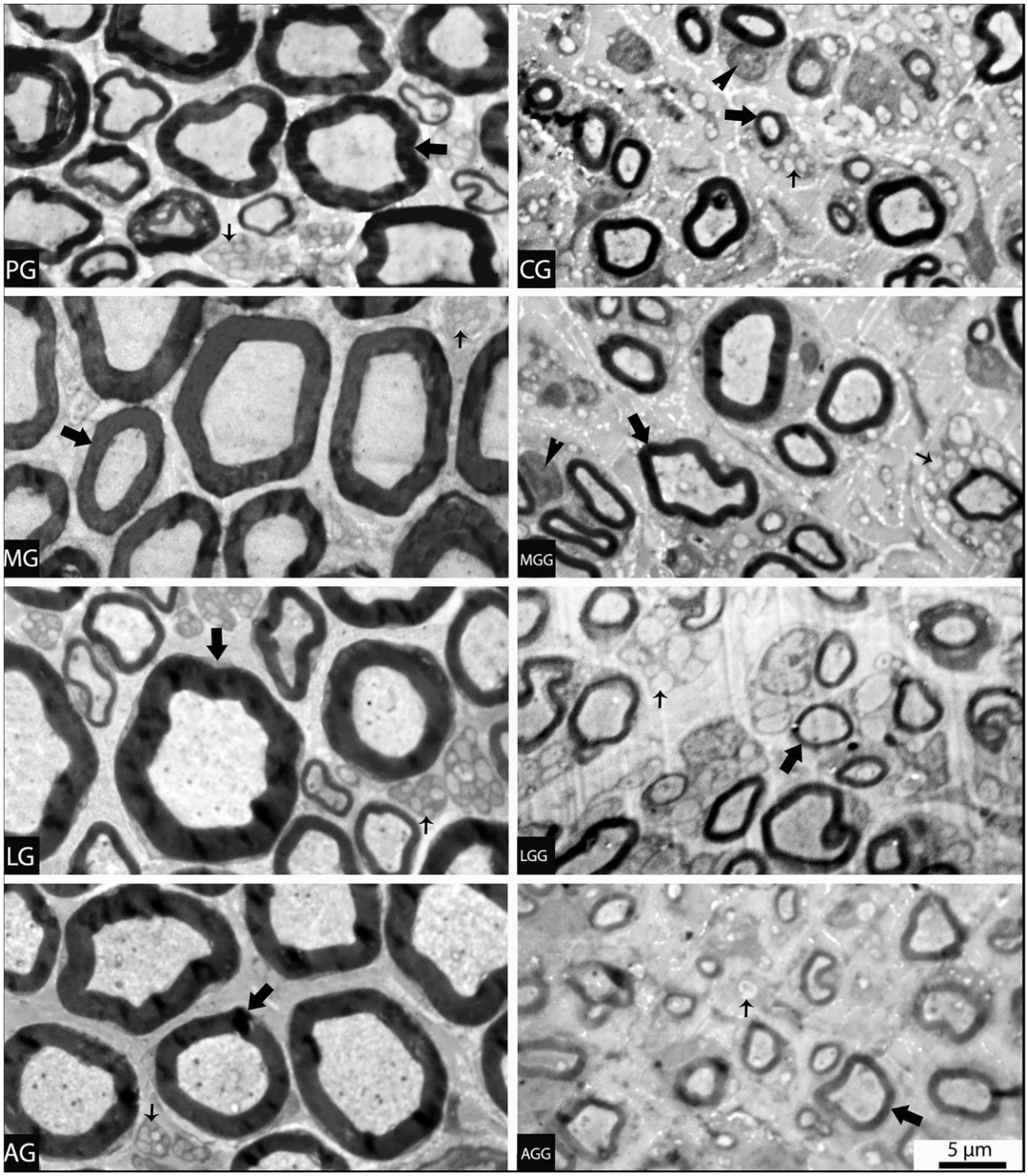

Fig. 8. Histopathological electron microscopic images of the gap and control groups (thick arrows indicate myelinated axons, thin arrows show unmyelinated axons, arrowheads indicate the nucleus of Schwann cells).

3.3.3. Amplitude and latency

From a statistical point of view, with the alcar treatment significant differences in amplitude and latency occurred between PG and AGG, CG and AG ($p<0.01$), whereas between PG and AG, CG and AGG there were no significant differences ($p > 0.05$).

4. Discussion

Peripheral nerve injury leads to partial or total loss in the motor, sensory and autonomic function. This process ends up with degeneration of nerve fiber and cell death. Therefore, the peripheral nervous system injuries can lead to serious disability (Navarro et al., 2007). Axonal regeneration is possible following the axonal injury. In this context, neuroprotective agents may increase Fig. 8. Histopathological electron microscopic images of the gap and control groups (thick arrows indicate myelinated axons, thin arrows show unmyelinated axons, arrowheads indicate the nucleus of Schwann cells). The aim of the present study is to research new treatments for peripheral nerve injury by using melatonin, alcar and leptin using by updated unbiased methods in stereology and with electron microscopy. At this point; melatonin exhibits potent free radical scavenger effects compared to other antiox- idants since it is soluble in both water and lipids and can therefore easily access all tissues. It has also been shown to reduce neuronal damage in the brain in experimental ischemia-reperfusion models (Poeggeler et al., 1994; Reiter et al., 2009; Sayan et al., 2004). Melatonin exhibits these antioxidant effects through its high binding potential to hydroxyl and peroxyl radicals, which has a direct impact on the independent receptors of toxic radicals and stimulates enzymes which inactivate the free radicals mediated by receptors (Atik et al., 2011). For example, the administration of melatonin before reperfusion has been shown to exert neuro-protective effects by preventing a decrease in levels of superoxide dismutase (SOD), one of the main antioxidants in the peripheral nervous system, and increasing levels of mRNA, which is involved in SOD synthesis (Atik et al., 2011). Although, both mechanisms are not effective in physiological application level, they are effective in changing the circadian rhythm in the higher levels (Atik et al., 2011). This is because plasma melatonin levels increase 3 to 10 fold in the dark period compared to a bright environment (Odaci and Kaplan, 2009). Although both myelinated and unmyelinated axon numbers were higher in MGG than CG, the difference was not statistically significant. This may be due to physiological doses not being administered, as described above. Contact between the axon sprouts and Schwann cells during axonal regeneration initiates various Schwann cell activities, such as secretion of neurotrophic factors and stimulation of myelin sheath formation (Lundborg and Richard, 2003). In terms of EMG amplitude values, the significant difference observed between MGG and CG and the absence of a significant difference between MG and MGG indicates an increase in Schwann cell activation, and thus in degree of myelination, rather than an increasing number of myelinated axons due to the antioxidant properties of melatonin. This is because melatonin exhibits antioxidant effects by protecting DNA, membrane lipids and intracellular proteins against oxidative damage (Odaci and Kaplan, 2009). Considering that the structure of the myelin sheath of peripheral nerves consists of lipids and proteins and that represents the main target of free radical-induced lipid peroxida- tion of myelin (Garbay et al., 2000; Sayar et al., 2004). The absence of a significant difference between MG and MGG may perhaps be attributed to EMG amplitude values. However, we think that the reason why the increased degree of myelination among the groups in terms of the thickness of the myelin sheath

did not attain statistical significance was because physiological doses were not applied. Leptin, another neurohormone, plays a key role in regulating energy homeostasis in the body (Friedman and Halaas, 1998). Several studies have shown that the systematic administration of leptin increases sympathetic nerve activity (Cao et al., 1997 Haynes et al., 1997a,b). Many studies have focused on the mechanism involved in leptin's central effects on muscle, heart, lungs, liver, and the sympathetic nervous system (Campfield et al., 1995; Chen et al., 1999; Li et al., 2013). Leptin has also been shown to stimulate central neurons (Mark et al., 2009). In addition, leptin has an important role into the perineurium and endoneu- rium (Maeda et al., 2009). However in the present study, there weren't statistical differences between CG and LGG on the myelin thickness. In terms of the number of myelinated axons, the increased statistical difference between CG and LGG shows that leptin administration has a positive effect on the regeneration. Leptin administration also increases the expression of neuro- protective genes (Fernandez-Martos et al., 2012). Our previous data indicate that leptins enhance nerve growth factor (NGF) activity in the spinal cord cells, so the differences between CG and LGG may be attributed to these neuroprotective properties of leptin as a neurohormone (Aloe et al., 2014). At this point; it can be said that leptin treatment may be a workable therapeutic methodology that can reverse nerve injury associated deficiency in growth factors helps to prevent mitochondrial oxidative stress by increasing mitotic activity and it also reduces nerve loss after axotomy. Considering the effect of alcar; neuronal survival and regeneration are dependent on neurotrophic factors, and they are necessary for metabolic pathways that provide high energy, such as oxygen respiration. As a physiological peptide, alcar is an antioxidant, which exhibits its activities by binding nerve growth factor and enhancing neuronal responses. Alcar has also been reported to inhibit cell death by suppressing mitochon- drial oxidative stress after nerve damage, and to reduce nerve loss post-axotomy (Aloe et al., 2014). Alcar has been shown to have an increasing effect on neuronal regeneration after peripheral nerve injury (McKay-Hart et al., 2002). The systemic administration of alcar after primary nerve repair increases nerve regeneration both quantitatively and qualitatively. The sensory and motor innervation of the target organs is therefore positively affected (Wilson et al., 2010). However, the exact mechanism involved in the reduction of sensory neuron loss is still unclear. In particular, it performs this function by facilitating the transport of long chain free fatty acids through the mitochondrial inner membrane (Wilson et al., 2007). Additionally, it exhibits this neuroprotective effect by increasing the transportation and regeneration of acetyl groups, the activity of mitochondrial DNA and the binding of nerve growth factors (Hart et al., 2004). The statistical significant decrease in the myelinated axons number in AGG compared to CG is consistent with the literature and it occurred due to the alcar's antioxidant, energy supplier and neuroprotective properties which are mentioned previously. In terms of unmyelinated axon numbers, the increase in AG compared to CG is due to neuroregenerative effects, as described above. In addition, the close similarity between CG and AGG values shows the powerful neuroprotective and regenerative effects of alcar. At this point; our results showed that only alcar has a beneficial effect on the regeneration of unmyelinated axons. Briefly, alcar therapy has a beneficial effect on the regeneration and myelination process in the sciatic nerve of the rats. In our further peripheral nerve studies, we are planning to support our results by using regenerative markers. We believe that our findings will encourage the researcher to investigate the new ways.

Disclosure statement

The authors declare that there is no conflict of interest.

Ethics statements

The Animal Ethics

Committee of Ondokuz Mayıs University approved the protocol and appropriate measures were taken to minimize pain or discomfort of the animals by our study group. The experimental part of this study and stereological examination was performed at Ondokuz Mayıs University, Department of Histology and Embryology.

Acknowledgment

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