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12 January 2025

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Possible effects of some agents on the injured nerve in obese rats: a stereological and electron microscopic study

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Abstract

Objective: Axonal regeneration is possible following the axonal injury. In this context, neuroprotective agents may increase the axonal regeneration in the obese rats. Prevalence of obesity induced by some drugs and neurobiological diseases is increasing day by day. Briefly, main aim of the present study is to research new treatments after peripheral nerve injury in light of Melatonin (Mel), ALCAR (AC) and Leptin (Lep) using by updated unbiased methods in stereological and electron microscopic levels.

Material-Method: *Wistar albino* rats were randomly divided to nine equal groups: control (Cont), obese control (OG), obese group exposed sciatic nerve resection (Gap) (OGG), obese group injected 50 mg/kg/day of melatonin intraperitoneally (i.p.) for 21 days, (OMG), obese group injected melatonin i.p. with gap for 21 days (OMGG), obese group injected 1mg/kg/day of leptin i.p. for 21 days (OLG), obese group injected leptin i.p. for 21 days with gap (OLGG), obese group injected 50mg/kg/day of ALCAR i.p. for 21 day (OAG), obese group injected acetyl L carnitine i.p. for 21 days with gap (OAGG). Electromyography (EMG) procedures was performed shortly before the rats were sacrificed. Following the routine histological process in the level of light microscopy and electron microscopy, stereological analysis were performed to estimate the myelinated and unmyelinated axon numbers surface area, myelin thickness and myelin thickness/axon diameter ratio for each group. Data were analysed using statistical software package program SPSS 21.0.

Results: Considering the statistical results, no significantly differences between Cont, OB and OLG were found in terms of myelinated and unmyelinated axon number (p>0.05). Additionally it was observed that myelin sheat in Cont other groups administrated neuroprotective agents was thicker than groups with gap (p<0.01). Myelinated axon number of groups with gap showed a significantly increased compared to groups without gap (p<0.01). Furthermore, axon surface area

in OMGG decreased compared to OMG, OG and Cont (p<0.01). Also highly significant decrease in groups with gap compared to without gap ones (p<0.01).

Conclusion: The study has offered that axon surface area and myelin sheath thickness in the axonal regeneration were smaller compared to initial after gap formation. Especially, administration of Lep, Mel and AC as neuroprotective agents may provide a positive contribution to regeneration and myelination process in obese rats.

Key words: neuroprotective agents, obesity, nucleator, fractionator, electron microscopy.

1. Introduction

 The studies on peripheral nervous system (PNS) show that axonal regeneration is possible -a significant degree- after axonal injury (Yiu and He, 2006).When an axon is damaged, the distal portion undergoes to Wallerian degeneration and its myelin sheath is lost. Conversely, proximal segment undergoes a repair mechanism for regeneration. Although recovery rate varies depending on the damaged region in pathophysiological conditions, functional recovery usually occurs in case of preservation of axonal integrity (Allodi et al., 2012; Lundborg, 2004). During adulthood, peripheral nerve regeneration is seen at high potential. If axonal integrity can not be proctected, morphological alterations such as axon diameter and myelin sheath thickness of regenerated nerve fibres can not be reached to the normal levels which in the pre-trauma (Muratori et al.,2012). From this point of view, following the peripheral nerve injury especially in the case of axonal integrity distruption, it is required current studies about possible neuroprotective agents which potentionally increase the axonal regeneration.

 Obesity is one of the most important health problems increasing with modernization of the world. Obesity is a complex disease that occurs as a result of more energy uptake than expenditure and is characterized by chronic caloric imbalance. Many prominent factors such as genetic factors, environmental effects, metabolism, daily habits, cultural, and socioeconomic status play a key role in obesity onset. Prevalence of obesity which is induced by some drugs and neurobiological diseases is increasing day by day (Knechtet al., 2008; Naggert et al., 1997). The studies regarding peripheral nerve regeneration and role of obesity on this regeneration are still unsatisfactory. However, Bekar et al. (2014) suggested that obesity may affect regeneration negatively after injury. As support of this result, Miscio et al. (2005) repoted that obesity may

lead to peripheral neuropathy and decreasing on level of cAMP in the peroneal and tibial nerves. For these reasons we decided to study the role of obesity on peripheral nerve regeneration and evaluate treatment options.

Firstly melatonin was defined as a neurohormonal regulator for circadian rhythm and body temperature as well as an antioxidant, and neuroprotective effects that is mentioned in recent studies (Leon et al., 2005; Kaplan et al., 2011;17 Aygun et al., 2012; Shinozuka et al., 2013; López-Iglesias et al.2014). Melatonin secretion usually occurs at night and secretion continues until morning. It is synthesized from serotonin by a two-step pathway. When the melatonin **is** administered as exogenous, it easily passes trough the blood brain barrier reaching to high concentrations in the brain. Furthermore melatonin is characterized by having neuroprotective effects due to its some features such as free-radical scavenger, lipophilic and hydrophilic properties (Aygun et al., 2012)**.** Many studies suggested that melatonin significantly prevents DNA damage and neuronal apoptosis reducing oxidative stress (Feng et al., 2006; Pandi-Perumal et al., 2006). Therefore, melatonin administration in peripheral nerve regeneration which is related to nerve injury may be benificial because of exogenous melatonin administration significantly increases antioxidant defense mechanism (Chang et al., 2008).

In the recent years, leptin is also underlined as a neuroprotective agent besides known as a hormone regulating appetite. Leptin is mainly secreted from adipose tissue and most tissue including skeletal muscle, stomach mucosa, placenta, and chroid plexus (Sandoval and Davis, 2003; Auwerx and Staels, 1998). Level of plasma leptin concentrations is generally proportional to the mass of the fat tissue in the body (Wilding, 2001). The anti-obesity effect of leptin occured by reducing fatty acid oxidation and so mass of fat tissue reduces. Additionally, there are a lot of (Folch et al., 2012). Especially exogenous leptin administration reduces the neuronal damage following ischemia and stroke. Neuroprotective and neurogenetic effects of leptin on the neuronal and glial cells by means of development and increasing of neural stem cells are reported in some studies (Avraham et al., 2011; Perez-Gonzalez et al., 2011).

Acetyl-L-carnitine (ALCAR) is a potential mitochondrial antioxidant and especially shows a neuroprotective effect on neurodegenerative diseases such as Alzheimer, Huntington and Parkinson (Chrysostomou et al., 2013). Also this mitochondrial agent affects degree of stem cell differantiation and reduces adipogenesis while it stimulates osteogenesis and chondrogenesis (Lu et al., 2013). ALCAR can pass blood brain barrier by using cation / carnitine transporter OCTN2

and plays a key role in oxidation of free fatty acids (Smeland et al.,2012). It is reported that all neuroprotective effects of ALCAR is occured by increasing intracellular neurotrophic pathways or cholinergic neurotransmission (Bigini et al., 2002; Ori et al., 2002).

The studies show that recovery of peripheral nerve injury is possible using neuroprotective agents (Cheng et al., 2013). The main aim of the present study is to detect new treatments of peripheral nerve injury in the obese rats in light of neuroprotective agents using updated unbiased methods in stereological and electron microscopic levels.

2. Materials and Methods

2.1. Experimental Design

The present study was approved by the Experimental Animal Studies Ethics Committee of Ondokuz Mayis University with decision 2010/121 numbered. Subsequently, rats were obtained from the Experimental Animal Research and Application Centre of Medicine Faculty of Ondokuz Mayıs University (Samsun, Turkey). 54 female Spraque Dawley rats 8-10 weeks-old weighing 150-200 gr were used for this study.. Animals were randomly divided to nine equal groups consisting of control (Cont), obese control (OB), obese group exposed sciatic nerve resection (Gap) (OGG), obese group injected melatonin intraperitoneally (i.p.) for 21 days (50 mg/kg/day; Sigma-Aldrich, St Louis, MO, USA) (OMG), obese group injected melatonin i.p. with gap for 21 days (OMGG), obese group injected leptin i.p. for 21 days (1mg/kg/day; Sigma-Aldrich, St Louis, MO, USA) (OLG), obese group injected leptin i.p. for 21 days with gap (OLGG), obese group injected ALCAR i.p. for 21 days (50mg/kg/day; Sigma-Aldrich, St Louis, MO, USA) (OAG), obese group injected ALCAR i.p. for 21 days with gap (OAGG). All of rats were housed in plastic cage and maintained on appropriate temperature and humidity (22±2 °C, %50±5) and conditions of 12-h light/12-h dark cycle. Rats of each group, except Cont, fed with special diet containing 40% fat during 8 weeks, whereas rats of Cont were fed with standard rat chow (Altunkaynak et al., 2008).

2.2. Surgical Procedures

All surgerical procedures were conducted after i.p. injection of Ketamin (Ketasol ® 90 mg/kg, Richter Pharma AG, Weis, Australia) and Xylazin (Rompun ® 10 mg/kg, Bayer, Leverkusen,

Germany). Right legs of the rats were shaved and their gluteal region incised with a thin cut under deep anesthesia. Subsequently, biceps femoris muscle was dissected and sciatic nerve was removed. Collagen membrane (in Epigui, Riemser Inc., Germany) was placed about 10 mm above of nerve branch. After collagen membrane was fixed to the nerve, 5mm gap was occurred by means of resection. Finally gap region was completely surrounded and closed by collagen membrane in tube form. Thus, standard gap was provided for each rat (Figure 1).

2.3. Electrophysiological Analysis

The electromyography (EMG) procedures were performed shortly before the rats were sacrified at 90th day of operation for each group at Ondokuz Mayıs University Medical Faculty, Laboratory of Physiology Department. Nerve stimulation and recording were performed using a Power Lab 4SP device (AD Instruments, Sydney, Australia) and data were stored on the Scope software program (Version 3.7.2., AD instruments). The stimulating electrode was placed on nearly 10 mm distal of sciatic nerve. Also the EMG records were obtained using electrode, which placed 2.5 cm distance from stimulating electrode on gastrocnemius muscle (Figure 2). The stimulating voltages between 0.01 and 10 mV were applied. The latency and peak-peak amplitude of potential (p-p amplitude) were measured by using action potential curve. Finally the average of 3 action potential curve was evaluated for each rat and data were statistically compared to each other.

2.4. Tissue Processing

Following the electrophysiological analysis, rats were sacrified nerve samples were removed and fixed in 5 %gluteraldehyde solution to histological and stereological evaluations .After embedding samples were cut into 70 nm and 500nm-thickness sections using an ultramicrotome (Leica Ultracut UCT, Leica Microsystems GmbH, and Germany). Thin sections were stained with uranyl acetate- lead citrate for electron microscopic analysis and semi-thin sections were stained with % 1 toluidine blue for light microscopic examination. For electron microscopy, samples were evaluated using electron microscope (JEN-1010, JEOL, Tokyo,Japan) in Cavalieri Neuroscience Institute of Turin University, Italy in point of unmyelinated fibers, myelin thickness and axon area (Kaplan et al., 2013). For light microscopy, semi-thin sections were

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evaluated using stereology workstation (Stereoinvestigator 9.0, MicroBrieldField; Colchester,USA) in histology and embryology department of Ondokuz Mayıs University, Turkey in terms of myelinated fibers, myelin thickness and axon area (Kaplan et al., 2013; Altunkaynak et al., 2012).

2.5. Statistical Analysis

Data were analyzed using statistical software package program SPSS 21.0 for Mac (IBM Corporation). In comparison the groups, One Way ANOVA (Tukey's post-hoc test) was used and p-values < 0.05 were accepted as statistically significant

3. Results

3.1. Obesity Groups

3.1.1. Myelinated and Unmyelinated Axon Numbers

Stereological results showed no significantly difference between OG, Cont in terms of the number of myelinated and unmyelinated axons (p > 0.05). Although the number of myelinated axons highly significant increased in OGG, the number of unmyelinated axons highly significant decreased in OGG compared to OG and Cont (p < 0.01) (Figure 2, 3, 4) (Table 1).

3.1.2. Estimation of Surface Area, Myelin Thickness and Myelin Thickness/Axon Diameter Ratio

There was no significantly difference between Cont and OG in comparison of axon areas, myeline thickness and myelin thickness/axon diameter ratio (p > 0.05). But also it was observed a highly significant increase in OGG compared to OG and Cont in term of axon surface area and myelin thickness (p < 0.01). Conversely when the data of myelin thickness/axon diameter ratio were examined, there was a highly significant decrease in OGG compared to other groups (p < 0.01) (Figure 2, 3, 4) (Table 1).

3.1.3. Comparison of Amplitude and Latency Values

When the amplitude and latency values were compared among the groups, no significantly difference was found between Cont and OG ($p > 0.05$). There was a highly significant decrease in OGG group in comparison with the Cont and OG relating to amplitude values ($p < 0.01$). Also latency in OGG group was increased compared to Cont and OG ($p < 0.01$) (Table 1).

3.2. Melatonin Groups

3.2.1.Myelinated and Unmyelinated Axon Numbers

The stereological data showed no significantly difference in OMG compared with Cont and OG ($p > 0.05$) in terms of myelinated and unmyelinated axon number. On the other hand, myelinated axons number of OMGG high significantly increased compared to OMG, OG and Cont ($p < 0.01$). Contrarily, myelinated axons number of OMGG high significantly decreased compared to OMG, OG and Cont ($p < 0.01$). Although no significantly difference between OGG and OMGG in terms of unmyelinated groups ($p > 0.05$), a significantly decreasing in OMGG compared with OGG ($p < 0.01$) (Figure 2, 3, 4) (Table 1).

3.2.2. Estimation of Surface Area, Myelin Thickness and Myelin Thickness/Axon Diameter Ratio

There was no significantly difference in OMG compared with Cont and OG with reference to axon surface area ($p > 0.05$). Also axon surface area in OMGG decreased compared to OMG, OG and Cont ($p < 0.01$). Moreover, there was no significantly difference between OGG and OMGG ($p > 0.05$). Additionally it was observed that myelin sheat in Cont, OG, OMG was thicker than OMGG an OGG ($p < 0.01$). There was no significantly difference among Cont, OG, and OMG in comparison of myelin thickness and myelin thickness/axon diameter ratio ($p > 0.05$). Correspondingly, there was no significantly difference between OMGG and OGG ($p > 0.05$) (Figure 2, 3, 4) (Table 1).

3.2.3. Comparison of Amplitude and Latency Values

The stereological data represented that there was no significantly differences among Cont, OG and OMG in terms of latency and amplitude ($p > 0.05$). Also amplitude highly significant decreased in OG compared to other melatonin groups and Cont ($p < 0.01$). When the latency was

evaluated there was no significantly difference Cont and OB (p>0.05). Moreover, latency in OMGG significantly increased compared to OMG (p<0.05), highly significant increased compared to Cont and OB (p<0.01). When the difference between latency values of OMG and Cont, a highly significant increased in OMG compared to Cont (p<0.01). Similarly, the amplitude value was found as increased in OMG compared to OMGG (p<0.05) (Table 1).

3.3. Leptin Groups

3.3.1. Myelinated and Unmyelinated Axon Numbers

No significantly difference between Cont, OB and OLG was found in terms of myelinated and unmyelinated axon number (p>0.05). Also a highly significant increase in myelinated axon number of OLGG and contrarily a highly significant decrease in unmyelinated axon number of it was found in comparison with Cont (p<0.01). Additionally, a highly significantly increase in OLGG and OLG compared to OG in terms of myelinated axon number (p<0.01). When the unmyelinated axons examined, a highly significant decrease in OLGG was stand out compared to Cont, OG, OLG and OB (p<0.01) (Figure 2, 3, 4) (Table 1).

3.3.2. Estimation of Surface Area, Myelin Thickness and Myelin Thickness/Axon Diameter Ratio

Upon analyzing data of surface area, there was no significantly difference between Cont and OLG (p>0.05). But surface area of OB was significantly increased compared to OLG (p<0.01). Although any differences was not observed between OLGG and OG (p>0.05), a highly significant difference in surface area of OLGG compared to Cont, OB, OLG (p<0.01). Similarly, there was no significantly difference among Cont, OB and OLG when the myelin thickness was estimated (p>0.05). The myelin thickness in OLGG and OG was highly significant thinner than Cont, OB and OLG (p<0.01). At the same time, no significantly difference between OLGG and OG was found (p>0.05). Considering that the myelin thickness/ axon diameter ratio, there was no significantly difference between Cont and OLG (p>0.05). Additionally, the ratio in OLGG and OG was highly significant lower than Cont and OLG (p<0.01). When the ratio of OG was compared with OLG and OLGG, there was no significantly difference (p>0.05) (Figure 2, 3, 4) (Table 1).

3.3.3. Comparison of Amplitude and Latency Values

When the statistical data was examined, no significantly difference among Cont, OB and OLG in terms of amplitude and latency ($p>0.05$). The amplitude value of OLGG and OG was highly significant decreased compared to Cont, OB and OLG ($p<0.01$). Additionally, the amplitude value of OLGG was highly significantly increased in comparison with OG ($p<0.01$). Examining that the statistical analysis of latency values, a highly significant increase in OLG and OLGG was determined compared to Cont ($p<0.01$). Also there was no significantly difference between latency values of OB and OLG, OB and OLGG ($p>0.05$). But a highly significantly decreased in OLG compared to OLGG ($p<0.01$). Additionally, there was a highly significant increase in OG compared to other groups ($p<0.01$) (Table 1).

3.4. ALCAR Groups

3.4.1. Myelinated and Unmyelinated Axon Numbers

Stereological data of AC groups showed no significantly difference in OAG compared with Cont and OG in terms of myelinated and unmyelinated axon number ($p>0.05$). Furthermore, myelinated axons number of OAGG high significantly increased compared to OAG, OG and Cont ($p<0.01$). Similarly, no significantly difference between OGG and OAGG in terms of myelinated axon number ($p>0.05$). Contrarily, there was a highly significant decrease unmyelinated axons of OAGG compared with OAG, Cont and OG ($p<0.01$). However, unmyelinated axon number in the OAGG was highly significant increased compared to OGG ($p<0.01$) (Figure 2, 3, 4) (Table 1).

3.4.2. Estimation of Surface Area, Myelin Thickness and Myelin Thickness/Axon Diameter Ratio

In terms of surface area, no significantly difference between OG and OAC ($p>0,05$)were found. Moreover there was a highly significant decrease in OAGG compared to Cont and contrarily there was a highly significant increased in OAC compared to OAGG and Cont ($p<0, 01$). However, there was no significantly difference between OGG and OAGG ($p>0,05$).

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When examined the myelin thickness statistically, there was no significantly difference between Cont and OAC (p>0,05). Also there was a highly significant decrease in myelin thickness of OAGG compared to Cont and OG (p<0, 01). Additionally, there was a highly significant increase myelin thickness of OAC compared to OGG (p<0, 01).

When evaluated the myelin thickness/axon diameter ratio, no significantly difference between Cont and OAC pointed out (p>0,05).Similarly, there was no significantly difference between OG and OAC (p>0,05). But also when compared to OG, there was a highly significantly increase in myelin thickness/axon diameter ratio of OAGG (p<0,01). Contrary to this, there was no significantly difference between OGG and OAGG (p>0,05). Additionally, there was a highly significant increased in OAGG compared to OAC (p<0,01) (Figure 2, 3, 4) (Table 1).

3.4.3. Comparison of Amplitude and Latency Values

Group	Myelinated Axon numbers	Unmyelinated Axon numbers	Axon Area (μm^2)	Myelin Thickness (μm)	Amplitude (mV)	Latency (s)
	Mean± SE	Mean± SE	Mean± SE	Mean± SE	Mean± SE	Mean± SE

Statistically, no significantly difference between amplitude values of Cont and OAC (p>0,05)were found. However, there was a highly significant decrease in OAGG compared to Cont and OG (p<0,01). Additionally, there was a highly significant increase in OAC compared to OGG and OAGG (p<0,01).

In terms of latency values, there was highly significant increase in OAGG compared to Cont and OG (p<0,01). Similarly, there was highly significant increase in OAC compared to Cont and OG (p<0,01). But no significantly difference between OAGG and OAC when evaluated the latency values (p>0,05). Also latency values in OGG was highly significantly higher than the OAC and OAGG (p<0,01) (Table 1).

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CONT	7549,83± 48,61	17228,49±485,43	17,39±1,36	1,19±0,06	46,02±1,22	1,34±0,02
OG	78872,76±74,86	16748,49±176,25	30,57±1,040	1,22±0,03	47,89±2,41	1,20±0,06
OGG	11479,69±181,09	8600,47±298,11	5,97±0,12	0,65±0,01	8,82±0,80	2,58±0,10
OMG	7669,35±116,70	15717,46±306,62	30,29±1,57	1,20±0,04	45,19±3,43	1,87±0,14
OMGG	9762,12±220,55	7154,59±252,22	6,23±0,29	0,65±0,01	33,75±2,30	2,46±0,19
OLG	7495,91±98,59	17709,26±149,39	26,02±0,34	1,12±0,01	43,45±0,74	1,72±0,13
OLGG	9102,62±275,61	6579,22±233,21	6,86±0,27	0,64±0,00	31,48±2,54	2,12±0,15
OAG	7838,02±264,34	17055,71±255,25	32,89±1,58	1,36±0,04	26,2±0,97	2,05±0,05
OAGG	11124,03±218,69	14589,19±166,67	6,58±0,11	0,67±0,00	47,81±2,06	2,27±0,20

Table 1. Mean values for myelinated, unmyelinated axon numbers, axon area, and myelin thickness obtained from the stereological estimation and mean values for amplitude and latency obtained from electromyography analysis.

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). Even though recovery of peripheral nerve system was considered as easier than recovery of central nervous system, the nerve regeneration mechanism it is still not fully understood. Although the role of several molecules in these mechanisms is still unknown, especially interactions between the different moleucles are not clear (Allodi et al., 2012).

Obesity is a serious health problem which has a high incidence in both of developed and developing countries. Especially the high incidence in children and young adults draws attention to the increasing health risks (Wang et al., 2002; Das, 2010). Although the low sympathetic activities of some region in the body such as skeletal muscle and adipose tissue were considered as a risk factor, there is not enough literature about effect of obesity on peripheral nerve

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regeneration (Davy and Orr, 2009). The present study allows discussing about the role of obesity with neuroproctective agents on peripheral nerve injury. Following the gap in the sciatic nerve, the degree of regeneration was observed. Gap style is suitable model in terms of a possible regeneration. But in this case, the main problem is non-standardization of injury. Therefore, in our study, 5 mm gap created in all experimental procedures to minimize these doubts.

There are a lot of studies about functional and morphological approaches in the injury model (Luís et al., 2007; Tos et al., 2008; Piskin et al., 2009; Kaplan et al., 2010; Gärtner et al., 2012). But none of them provide sufficient information regarding the nerve function itself. This evaluation gives us information about different stage of degeneration and so there is no correlation among them (Kanaya et al., 1996; Varejao et al., 2004). From this view point, the combination of electrophysiological, histological and stereological methods was performed in the present study in order to determine the degree of regeneration.

Axonal regeneration is one of the unique biological processes including cellular repair rather. The essential purpose of regeneration is recycyling the initial morphology of each cell. The various events such as proliferation of fibroblasts, endothelial cells and Schwann cells in the local wound healing regions are necessary for this recycling. This peripheral gap region is characterized with exudation, increased cell proliferation and collagen synthesis (Lundborg, 2004). Axonal regeneration rate is slower in nerve with gap. GAP formation also significantly increase the number of axons sprouts (Brushart, 2011). After the injury, within a few hours, a lot of ends extending from the proximal to the distal of axon begin to appear. This axon sprouts degenerate and replace the permanent sprouts. Each axon in distal Schwann cell tube continues to collaterals branching. Also this process results with axon formation in the regenerating nerve (Geuna et al., 2009).

Schwann cells are essential structural and functional cells in the regeneration and contribute to the axonal regeneration via consecutive three different ways. To promote the axonal growth, this process consists of secretion of several neurotrophic factors, creating roof of regenerating axons by expression of adhesion molecules and formation of myeline for transmission (Geuna et al., 2009; Wang et al., 2012). The loss of contact between the axon and the Schwann cells are considered a signal for inducing axonal regeneration in three different ways (Geuna et al., 2009).It was suggested that adipose derived stem cells increase the regeneration in peripheral nerve with gap and stimulate the axonal growth in proximal segment, Schwann cell proliferation

in distal segment (di Summa et al., 2010; Erba et al., 2010). This process provides an advantage in decrease time between injury and treatment time (Terenghi et al., 2009). Obesity groups in our study, the increase of adipose tissue surrounding epineurium may affect epineural connective tissue elements. On account of this, axonal regeneration in obese group may be higher compared to Cont. Axonal surface area and myelin thickness was parameters regarding maturity level of nerve fiber (Varejao et al., 2004). During the regeneration, axon sprouts expands, mature and reach to approximately normal diameter in initial (Lundborg, 2004). Regarding this aspect, statiscal difference between Cont and OGG, OGG and OB in terms of myelin thickness and axon surface area is an expected result. Because surface area and myelin thickness of axon sprouts following gap formation is smaller than intial and there is no entirely maturation. In this context, coinciding of myelin thickness and degree of myelination with amplitude and latency values confirmed our foresights about myelin thickness of regenerated axons. Regarding the degree of myelination, increasing statistical difference between Cont and OGG, OG and OGG in myelin thickness/ axon diameter ratio indicated that decrease in axon diameter was higher than myelin thickness. During the regenerarion, axon sprouting from myelinated nerve stimulates Schwann cell in distal segment to form the myelin sheat. Unmyelinated axons initially regenerated to distal myelinated nerve fiber but also they remain as unmyelinated fibers (Lundborg, 2004). Following the gap formation, the lot of unmyelinated axon degenerates, some of them don't reach to distal part and thus unmyelinated axon number decrease compared to pre-regeneration period. Therefore Statiscal differences between Cont and OGG, OG and OGG may be regarding this decrease.

Melatonin, as a neuroproctetive agent, has a strong free radical scavenging effect (Poeggeler et al., 1994; Sayan et al., 2004). Melatonin shows the antioxidant effect by two ways. First mechanism depends on direct effect on toxic radicals because, melatonin has high affinity for these radicals. The second mechanism depends on enzymes stimulation via receptors (Atik et al., 2011). The decreasing statistical difference between OG and OMG in terms of myelinated and unmyelinated axon number revealed that melatonin shows a different effect in obesity. As known, melatonin reduces the white adipose tissue mass and prevent the gaining weight. Also it increases brown adipose tissue formation and oxidative phosphorylation activity of mitochondria and shows positive effect on metabolic activity of the tissue. Regarding this point of view, melatonin may show antiobesity effect (Tan et al., 2011; Terrón et al., 2013). As a result of this

effect of melatonin in obes rats, decreased white adipose tissue mass and increased metabolic activity in brown adipose tissue may decrease the energy required for regeneration and population of stem cell in white adipose tissue.

Additionally, during the axonal regeneration, contact between axon sprout and Schwann cell initiates a lot of activities of Schwann cells such as secretion of neurotrophical factors, stimulation of myeline sheat (Lundborg, 2004). In terms of EMG amplitude values, increasing statistical difference between OG and OMGG, also no statistical difference between OMG and OMGG is considered due to increase of Schwann cell activation by melatonin rather than increased the number of myelinated axons. Whereat, melatonin may increase the myelinization degree. When the myelin sheat structure consists of lipid and protein considered, in terms of EMG amplitude values, no difference between OMG and OMGG is explainable (Garbay et al., 2000; Sayan et al., 2004). In this context, this statistical result is due to lack of physiological doses.

High fat diet leads to constant and increased leptin level. So, increased plasma leptin level is regarding to leptin resistance (Frederich et al., 1995; Banks et al., 2006). The essential effect of leptin focused on increase the feeling of satiety and decrease body weight. Therefore, gradually increasing leptin resistance occurs in normal experimental animals which fed with high fat diet (Haffner et al., 1996). Several studies reported that administration of leptin 122 increases the sympathetic activity (Cao et al., 1997; Haynes et al., 1997a; Haynes et al., 1997b). Contrarily, some studies show that leptin administration can suppress nervous system. In this case, it can be said that central nervous system response of obes rats to systemic leptin administration is different than others. However, in case of restriction of food intake, enhancing sympathetic activity by leptin shows that this different effect in obes rats is reversible (Lu et al., 1998). Fedding of rats with high fat diet during the experimental process showed that mentioned reversible effect was eliminated. Systemic leptin administration stimulates factors that inhibit the proliferation of all cell types in adipose tissue in normal rats and increase the number of preadipocytes to increase the expansion of adipose tissue mass (Harris, 2013). When the examined the data of EMG amplitude values, increasing statistical difference between OGG and OLGG indicates the possible increased Schwann cell population depending on density of stem cells and hence increased myelinization degree. In terms of myelin thickness / axon diameter there is no statistical difference between OGG and OLGG in the present study. This case

indicates that exogen leptin administration in the obes rats lead to decrease in loss of myelin thicknes in proportion to axon diameter. These results confirm our views regarding increased myelinization degree in obes groups.

Neuronal survival and regeneration is dependent on neurotrophic factors and needs metabolic pathways which provide a high level of energy such as aerobic respiration. ALCAR, as a physiological peptide, is an antioxidant and enhances the nerve growth factor 123 and neuronal response. After the nerve injury, it prevents the active cell death by suppressing mitochondrial oxidative stress and it also reduces nerve loss after axotomy (Wilson et al., 2010). Also it was reported that AC increased the regeneration after peripheral nerve, independently of the effect on neuronal survivor (McKay Hart et al., 2002). After the primary nerve repair, AC administrated systematically, increased the nerve regeneration as both qualitative and quantitative and as a result of this, positive effects on sensory and motor innervation of the target organ occur (Wilson et al., 2010). But also the whole mechanism in reducing of sensory neuron losses is not clear. It has been suggested that AC meet the requirement of neuronal energy after nerve injury. Especially, this function performs by facilitating the transport of long-chain free fatty acids through the inner membrane of mitochondria (Wilson et al., 2007).Additionally, it has neuroprotective affect such as accelerating the transport and renewal of acetyl grous, increase of mitochondrial DNA activity, raising the affinity of nerve growth factor by means of upregulating the NGF expression (Hart et al., 2004).There is no significantly difference between OGG and OAGG in terms of myelinated axon number and this result showed that AC has no effect like mentioned above in obesity.Also the statistical difference between OGG and OAGG in terms of unmyelinated axon number may be related to neuroprotective effects of AC. Eventhough there is no significant difference between nonobese groups and unmyelinated axon number in nonobese groups was closed to number of groups without gap, As a result of this, neuroprotective and regenerative effect of AC was considered.

Briefly, administration of Lep, Mel and AC may benefit to regeneration and myelination process in obese rats. We believe that our findings will encourage the researcher to investigate the new ways.

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.

Conflict the Interest Statement

The authors declare that there is no conflict of interest.

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