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The Amelioration Effects of Ankaferd Blood Stopper, Platelet Gel and Momordica charantia on Peripheral Nerve Injury in the Rats: A Stereological and Ultrastructural Study

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Abstract

Objective: Peripheral nerve injuries are commonly encountered in clinical practice. Peripheral nerve injuries most commonly resultin serious problems affecting quality of life. The present study is designed to research the possible neuroprotective effects of Ankaferd blood stopper (ABS), Platelet-rich plasma (PRP) and *Momordica charantia* (MC) on regeneration using unbiased stereological techniques.

Material-Method: 30 female 8-10 weeks-Sprague Dawley rats were randomly divided into five equal groups; control (CG), the group exposed to sciatic nerve resection (Gap) (CGG), additionally following the surgical process ABS, MC and PRP were injected into collagen tube (ABSG, MCG, PRPG). Nucleator and fractionator methods were used to estimate the number of myelinated and unmyelinated axons, axon area, and myelin sheath thickness. Also, an electron microscopic evaluation was performed.

Results: Regarding the number of myelinated axons, significant increases were found in the ABSG, MCG and PRPG compared to CG ($p<0.05$).

Conclusion: The protective effects of ABS, PRP, and MC on peripheral nerve regeneration in experimental models in rats were shown using electrophysiological and stereological assessment parameters.

Introduction

A traumatic peripheral nerve injury is one of the reasons for disabilities worldwide. Accidents usually cause various degrees of damage to nerve fibers in daily life. The damage in the nerve fiber's distal or/and proximal regions brings significant histopathological alterations. Although various molecules that play a key role in axonal regeneration are known, interactions among glial cells, axons and extracellular molecules have still not been fully explained. The extent of damage at the axonal level is the principal factor in determining the recovery rate and duration. While recovery rate changes depend on pathophysiological alterations in the damaged area, full functional recovery occurs as long as axonal integrity is maintained (Robinson, 2000; Lundborg, 2003; Allodi et al., 2012).

It is known that peripheral nerve injuries are commonly encountered in clinical practice. Peripheral nerve injuries most commonly result in serious problems affecting quality of life. When axonal continuity is destroyed, Wallerian degeneration occurs in the distal segment, and the degeneration progresses toward the proximal area. The regenerating axons try to fill a long gap in the distal stump to ameliorate functional loss without surgical intervention; also, there is a spontaneous axonal regeneration. On the other hand, nerve auto-graft implantation is the most common repair mechanism for long gaps in clinical practice (Alluin et al., 2009).

The trauma caused by peripheral nerve injury leads to damage to nerve fibers. Following a nerve injury, morphological and metabolic changes occur in the proximal and distal segments of the damaged area and perikaryon (Geuna et al., 2009). These alterations consist of an increase in the volume of perikaryon, the disintegration of Nissl bodies in the cytoplasm (chromatolysis) and nuclei in a peripheral region. In

the chromatolysis reaction, neurons change from "signaling mode" to "growth mode." In this regard, protein synthesis is prone to producing the necessary materials for axonal reconstruction instead of producing substances that will join the neurotransmitter structure (Lundborg, 2003).

Generally, although the recovery rate in the peripheral nerve injury changes depending on pathophysiological alterations in the injury region, full functional recovery occurs as long as the axonal integrity is protected (Robinson, 2000; Lundborg, 2003; Allodi et al., 2012). As is known, the high regeneration potential of the peripheral nerve in the adult period continues. However, when the axonal integrity cannot be maintained, it has been reported that regenerated nerve fibers could not reach the normal levels pre-trauma in terms of some parameters, such as axon diameter and myelin sheath thickness (Muratori et al., 2012).

The platelet-rich plasma (PRP) is autologous plasma containing normal platelets (Pietrzak and Eppley, 2005; Eppley et al., 2006). When the PRP is injected into connective tissue, it activates the platelets and initiates the formation of a fibrin skeleton by contacting the tissue thromboplastin (Arnoczky, 2011). Cytokines and growth factors have been stored in α granules of platelets. These cytokines and growth factors, which consist of platelet factor 4, interleukin and platelet-derived endothelial growth factor and platelet-derived growth factor, are activated in the first 10 minutes following coagulation via activation of the platelets. Thus, PRP is believed to possess regeneration capacity (Piskin et al., 2009). The platelet-derived growth factor enhances the osteoblasts in the bone, and the platelet-derived endothelial growth factor enhances the mitotic activity of endothelial cells (Sommeling et al., 2013). However, there is no adequate literature on the effect of PRP on nerve regeneration. It has been observed that PRP increased the functional recovery in the facial nerve after experimental peripheral nerve injury (Farrag et al., 2007). Nevertheless, it has been demonstrated that PRP has no healing effect on the axonal regeneration of the sciatic nerve (Piskin et al., 2009).

Ankaferd blood stopper (ABS) can affect the levels of crucial proteins and factors responsible for metabolic pathways, apoptosis, and angiogenesis (Haznedaroglu et al., 2012; Barkun et al., 2013). Compared with the other haemostatic agents, it has been reported that ABS provided an acceptable recovery in peripheral nerve injury (Pampu et al., 2013). However, studies about the effects of ABS on functions and parameters of peripheral nerve injury were required.

Momordica charantia (MC) has a lot of biological effects such as immune enhancement, anti-oxidation and anti-cancer. However, the potential protective effects of MC on stroke damage are not clear. Also, MC had a neuroprotective effect against intra-cerebral hemorrhage by inhibiting the JNK3 signaling pathway (Duan et al., 2015). However, no study or investigation of the possible protective effect of sciatic nerve injury has been conducted in the literature. In this regard, studies that examine the effects of neuroprotective agents in promoting possible axonal regeneration in case of disruption in axonal integrity were required. Therefore, this study will reveal the possible neuroprotective effects of ABS, PRP and MC on regeneration using unbiased stereological techniques.

Materials and Methods

Study groups

The Experimental Animal Studies Ethics Committee of Ondokuz Mayis University approved the study under approval No. 2010/121. Rats were obtained from the Experimental Animal Research and Application Center of the Ondokuz Mayıs University Medical Faculty (Samsun, Turkey). Thirty female Sprague Dawley rats aged 8-10 weeks weighing 150-200 g were used. Animals were randomly divided into five equal groups: control (CG) the group exposed to sciatic nerve resection (Gap) (CGG). Additionally, following the surgical process, 1 ml of ABS (Ankaferd İlaç Kozmetik Üretim A.Ş.), MC extract and PRP were injected into collagen tube for ABS, MC and PRP groups (ABSG, MCG, PRPG). For the preparation of MC extract, mature MC was identified by the Botanical Research Institute of Ondokuz Mayis University. All rats were housed in plastic cages and maintained in appropriate temperature and humidity conditions (22±2 °C, 50±5%) in a 12-h light/12-h dark cycle.

PRP preparation

2ml of peripheral venous blood was collected into the conical tubes (15 mL; BD Falcon) that contained either 1 mg/mL ethylene di-amine tetra-acetic acid disodium (EDTA; Wako Pure Chemical Industries, Ltd.) as an anticoagulant under the anesthesia. The isolate plasma tubes were centrifuged at 1100 rpm for 7 min at 20 ^{o}C in the ALC-CG 130 device (CG 130, Monzeerre, Italy). The platelet-rich plasma was gently transferred to a tube, and hematological values were measured. The concentration of platelets was 1,100.000 – 1,300.000 per mm^3. The obtained plasma (the PRP) was centrifuged again at 3000 rpm (10 min, 20°C) to provide precipitation of the platelets at the bottom of the tube. Approximately 200 µl of platelets were obtained at the end of this process. Lastly, at the hematological measurements, 2,500.000 -3,000.000 platelets per mm^3 were found. 20 µl calcium glibionate was added for PRP gel formation (Yu et al., 2011).

Dissection of peripheral nerves and electrophysiological analysis

All surgical procedures were performed after i.p. injection of ketamine (Ketasol ® 90 mg/CGG, Richter Pharma AG, Weis, Australia) and Xylazine (Rompun ® 10 mg/CGG, Bayer, Leverkusen, Germany). The right legs were shaved, and the gluteal regions were incised with a thin incision under deep anesthesia. The sciatic nerve was removed after dissection of the biceps femoris muscle. A collagen membrane (in Epigui, Riemser Inc., Germany) was placed approximately 10 mm above the nerve branch. Resection resulted in a 5-mm gap. The gap region was then surrounded and closed by a collagen membrane in tube form. Standard gaps were thus produced for each rat. EMG procedures were performed before each rat was sacrificed on the 90th day at the Ondokuz Mayıs University Medical Faculty Physiology Department laboratory. Nerve stimulation and recording were performed using a Power Lab 4SP device (AD Instruments, Sydney, Australia), and data were stored on Scope software (Version 3.7.2., AD instruments). The stimulating electrode was placed approximately 10 mm distally to the sciatic nerve. EMG records were obtained using an electrode placed 2.5 cm from the stimulating electrode on the

gastrocnemius muscle, and stimulating voltages were applied between 0.01 and 10 mV. The latency and peak-peak amplitude of potential (p-p amplitude) were measured using action potential curves. The average of three action potential curves was analyzed for each rat, and the data among the groups were compared statistically.

Histological and stereological procedures

Each rat was sacrificed, and nerve tissues were removed after electrophysiological analysis and fixed in a 5% glutaraldehyde solution for histological and stereological evaluations. After blocking, 70 nm and 500 nm-thick sections were obtained using an ultramicrotome (Leica Ultracut UCT, Leica Microsystems GmbH, 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 the electron microscope (JEN-1010, JEOL, Tokyo, Japan) at the Cavalieri Neuroscience Institute of Turin University, Italy, in terms of unmyelinated fibers, myelin thickness and axon area (Figure 1) (Kaplan et al., 2013).

For light microscopy, semi-thin sections were evaluated using a stereology workstation (Stereoinvestigator 9.0, MicroBrieldField; Colchester, USA) in the Department of Histology and Embryology of the Ondokuz Mayıs University Department of Histology and Embryology in terms of myelinated fibers, myelin thickness and axon area using nucleator and fractionator method. At the method application, 60 µm x 60 µm of grid size and 25 µm x 25 µm of frame size were used. The pilot study was performed to obtain an acceptable *coefficient error (CE)* (Figure 2) (Altunkaynak et al., 2012; Kaplan et al., 2013).

Statistical analysis

All data are expressed as mean ± standard error mean using SPSS 21.0 for Mac (IBM Corporation) software. Statistical significance was calculated using a one-way ANOVA (Tukey's post-hoc test). Significance was set at $p < 0.05$ or $p < 0.01$.

Results

Control groups

Histopathological evaluation

When evaluating the light and electron microscopic images of CG, mono fascicles in the nerve sections were highlighted. The connective tissue of the peripheral nerve seemed healthy. Most myelinated axons and uniform boundaries of myelin sheaths were generally seen. Additionally, healthy Schwann cells surrounding the axons with their euchromatic nuclei cytoplasmic boundaries were observed. Also, vascularisation in the connective tissue of the peripheral nerve was determined. In the CG, there were a lot of small-diameter myelinated axons. Also, there were a lot of macrophages in CG's connective tissue. Also, Schwann cells had normal structures in the CG (Figures 3 and 4).

Number of myelinated axons

In terms of number of myelinated axons, a significant increase was found in the CGG compared to CG ($p<0.01$) (Figure 5).

Number of unmyelinated axons

In terms of the number of unmyelinated axons, a significant increase was found in the CG compared to CGG ($p<0.01$) (Figure 5).

Axon area

In terms of the number of axon areas, a significant increase was found in the CG compared to CGG ($p<0.01$) (Figure 6).

Myelin sheath thickness

In terms of the number of myelin sheath thicknesses, a significant increase was found in the CG compared to CGG ($p<0.01$) (Figure 6).

The ratio of myelin thickness to axon diameter

In terms of the number of the ratio of myelin thickness to axon diameter, a significant increase was found in the CG compared to CGG ($p<0.01$) (Figure 6).

Amplitude

In terms of amplitude values, a significant decrease was found in the CGG compared to CG ($p<0.01$) (Figure 7).

Latency

In terms of latency values, a significant increase was found in the CGG compared to CG ($p<0.01$) (Figure 7).

ABS groups

Histopathological evaluation

The presence of many small-diameter myelinated axons in the nerve tissues of the rats, which were more apparent in the CG, was remarkable. Sparser localizations of unmyelinated axons located among myelinated axons were evident when compared with groups with no gap. In the endoneurium, there were more and larger vascularization and macrophages than in groups with no gap. Schwann cells had a normal appearance (Figure3 and 4).

Number of myelinated axons

A significant increase was found in the ABSG compared to CG regarding the number of myelinated axons ($p<0.01$). However, no significant difference was found between CGG and ABSG ($p>0.05$) (Figure 5).

Number of unmyelinated axons

A significant decrease was found in the ABSG compared to CG regarding the number of unmyelinated axons ($p<0.01$). Additionally, a significant increase was found in the CGG compared to ABSG ($p<0.01$) (Figure 5).

Axon area

When comparing the axon area of ABSG and CG, a significant decrease was found in the ABSG compared to CG in terms of myelinated axon area ($p<0.01$). No significant difference was found between the CGG and ABSG groups ($p>0.05$) (Figure 6).

Myelin sheath thickness

In terms of myelin sheath thickness, a significant decrease was found in the ABSG compared to CG in the myelinated axon area ($p<0.01$). There was no significant difference in the ABSG and CGG ($p<0.05$) (Figure 6).

The ratio of myelin thickness to axon diameter

In terms of the ratio of myelin thickness to axon diameter, a significant increase was found in the ABSG compared to CG ($p<0.01$). No significant difference was found between the CGG and ABSG groups ($p>0.05$) (Figure 6).

Amplitude

A significant decrease was found in the ABSG compared to CG ($p<0.01$) regarding amplitude values. A significant increase was found in the ABSG compared to CGG ($p<0.01$) (Figure 7).

Latency

Regarding latency values, a significant decrease was found in the ABSG compared to CG ($p<0.01$). No significant difference was found between the CGG and ABSG groups ($p>0.05$) (Figure 7).

PRP Groups

Histopathological evaluation

The presence of many small-diameter myelinated axons in the nerve tissue samples of PRPG, which were more apparent in the control group, was remarkable. Sparser localizations of unmyelinated axons located among myelinated axons were evident when compared with groups with no gap. There was

more vascularization and macrophages in the endoneurium than in the groups with no gap. Schwann cells had normal structures (Figures 3 and 4).

Number of myelinated axons

A significant statistical increase in the PRPG was found compared to CG regarding the number of myelinated axons ($p<0.01$). A significant increase in the CGG was found compared to CG ($p<0.01$) (Figure 5).

Number of unmyelinated axons

A significant decrease in the PRPG was found compared to CG regarding the number of unmyelinated axons ($p<0.01$). A significant increase was found in the CGG compared to PRPG groups ($p<0.01$) (Figure 5).

Axon area

There was a significant decrease in the PRPG compared to CG groups regarding myelinated axon area ($p<0.01$). On the contrary, no significant difference was found between the CGG and PRPG groups ($p>0.05$) (Figure 6).

Myelin sheath thickness

Regarding myelin sheath thickness, an extremely significant statistical difference was found between the CG and PRPG groups ($p<0.01$). An extremely significant difference was found between the CGG and PRPG groups ($p<0.01$) (Figure 6).

The ratio of myelin thickness to axon diameter

Regarding the ratio of myelin thickness to axon diameter, a significant increase was found in the PRPG compared to CGG ($p<0.01$). No significant difference was found between the CGG and PRPG groups ($p>0.05$) (Figure 6).

Amplitude

Regarding amplitude values, a significant decrease was found in the PRPG compared to CG ($p<0.01$). In addition, a significant decrease was found in the CGG compared to PRPG ($p<0.01$) (Figure 7).

Latency

Regarding latency values, a significant increase was found in the PRPG compared to CG ($p<0.01$). In addition, a significant increase was found in the CGG compared to PRPG ($p<0.01$) (Figure 7).

MC Group

Histopathological evaluation

The presence of a great number of small-diameter myelinated axons in the nerve tissue samples of the MC group was remarkable. Sparser localizations of unmyelinated axons located among myelinated axons were evident when compared with groups with no gap. There was more vascularization and macrophages in the endoneurium than in the groups with no gap. Schwann cells had a normal appearance (Figures 3 and 4).

Number of myelinated axons

A significant increase was found in the MCG compared to CG regarding the number of myelinated axons ($p<0.01$). In addition, a significant increase was found in the CGG compared to CG groups ($p<0.01$) (Figure 5).

Number of unmyelinated axons

A significant decrease was found in the MCG compared to CG regarding the number of unmyelinated axons ($p<0.01$). In addition, a significant decrease in the MCG was found compared to CGG ($p<0.01$) (Figure 5).

Axon area

A significant decrease was found in the MCG compared to CG regarding myelinated axon area ($p<0.01$). However, no significant difference was found between CGG and MCG groups ($p>0.05$) (Figure 6).

Myelin sheath thickness

In terms of myelin sheath thickness, a significant decrease was found in the MCG compared to CG ($p<0.01$). However, no significant difference was found between CGG and MCG groups ($p>0.05$) (Figure 6).

The ratio of myelin thickness to axon diameter

In terms of the ratio of myelin thickness to axon diameter, a significant increase in the MCG was found compared to CG ($p<0.01$). However, no significant difference was found between CGG and MCG groups ($p>0.05$) (Figure 6).

Amplitude

Regarding amplitude values, a significant increase in the MCG was found compared to CG ($p<0.01$). A significant increase was found in the MCG compared to CGG ($p<0.01$) (Figure 7).

Latency

Regarding latency values, a significant decrease in the MCG was found compared to CG ($p<0.01$). A significant decrease was found in the MCG compared to CGG ($p<0.01$) (Figure 7).

Discussion

Peripheral neuropathies cause partial or total losses in motor, sensory and autonomous functions, resulting in the disappearance of axon continuity, degeneration of nerve fibers and eventually cell death. Thus, peripheral neuropathies can cause serious injuries (Navarro et al., 2007). Mechanisms in nerve regeneration after nerve injury are still not fully understood. Although various molecules are shown to play important roles in these mechanisms, especially how they interact with each other is not obvious (Allodi et al., 2012). After nerve injury, Wallerian degeneration (WD) is the signature pathological process in peripheral nerve repair. Myelin removal is essential for axon regeneration to proceed after nerve injury. A complex interaction exists between macrophages, fibroblasts, SCs, and mast cells expressing inflammatory and anti-inflammatory cytokines during WD. However, mechanisms that terminate the inflammatory response are not well understood. Therefore, developing treatments that manipulate inflammatory responses would likely lead to enhanced recovery of patients after peripheral nerve injury (Stoll et al., 2002; Gaudet et al., 2011; Han et al., 2016).

ABS has important functions such as cellular reproduction, vascular endothelial growth production and antioxidant features (Akalin et al., 2014). ABS's basic mechanism of action depends on forming a capsuled protein network that forms a basis for erythrocyte aggregation (Aysan et al., 2010). In an experimental study using electrophysiological tests, ABS was shown to have a positive and improving effect on peripheral nerve functions (Pampu et al., 2013). Under the light of current literature approaches, the increasing statistical difference between CGG and KACG groups in terms of the number of both myelinated and unmyelinated axons can have resulted from the possible antioxidant and cell reproduction effect of ABS. ABS may have increased the reproduction of epineural fibroblasts, especially in terms of antioxidant function, and thus, it can have contributed significantly to regeneration. The increasing statistical difference between CGG and KACG groups regarding EMG amplitude values confirms that ABS increases myelination degree rather than macro formations such as myelin sheath formation and shows a micro antioxidant effect.

PRP is plasmas that contain much more intense thrombocytes than normal blood values. In addition to the coagulation factor, they contain a great number of growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor-beta beta (TGF-β). They show their primary effect with the joint interaction of growth factors they include (Eppley et al., 2006). Same doses but many growth factors applied together rather than only one growth factor over the physiological dose is a big advantage in tissue regeneration (Anitua et al., 2013). PRP application means

that platelet growth factors are also applied to the related tissue area because α granules in platelets contain many growth factors, including events such as cell reproduction, differentiation and chemotaxis. These factors have a critical significance in the stimulation and organization of wound healing and play a significant role in cellular events such as mitotic activity, chemotaxis, differentiation and metabolic activities (Piskin et al., 2009). The increasing statistical difference between CGG and PRPG groups regarding the number of both myelinated and unmyelinated axons shows that the regenerative factors of PRP are quite effective on nerve regeneration. It can increase mesenchymal stem cell migration and differentiation and the reproduction of epineural fibroblasts by increasing the vascularization in PDGF, VEGF and IGF-I regeneration areas. The increasing statistical difference between CGG and PRPG groups regarding EMG amplitude values shows that PRP increases the degree of myelination.

MC has been shown to have antidiabetic and neuroprotective features since it decreases lipid peroxidation or has free radical scavenger characteristics (Ansari et al., 2005; Malik et al., 2011; Gong et al., 2015). It has been suggested to provide antioxidant homeostasis by removing the toxicity of free radicals in the brain due to its free radical scavenger characteristic and to show a neuroprotective effect by decreasing oxidative stress (Myojin et al., 2008). The increasing statistical difference between CGG and MCG groups in terms of the number of both myelinated and unmyelinated axons may have resulted from the antioxidant effect of MC on nerve tissues. The increasing statistical difference between CGG and MCG groups regarding EMG amplitude values is associated with the increased myelination degree dependent on MC application. The lipid peroxidation decreasing effect of MC and its antioxidant effect may have increased myelination in regeneration.

In conclusion, in our study, the effects of ABS, PRP and MC on the experimental peripheral nerve regeneration of rats were shown in terms of electrophysiological and stereological assessment parameters, and we believe that our results will contribute to the literature significantly in terms of the validity of the methods used and the currency of the subject. The protective effects of ABS, PRP, and MC treatment on nerve regeneration should be supported by studies that explain the mechanism.

Declarations

Acknowledgment

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Conflict of Interests

The authors acknowledge that no competing financial interests exist.

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Figures

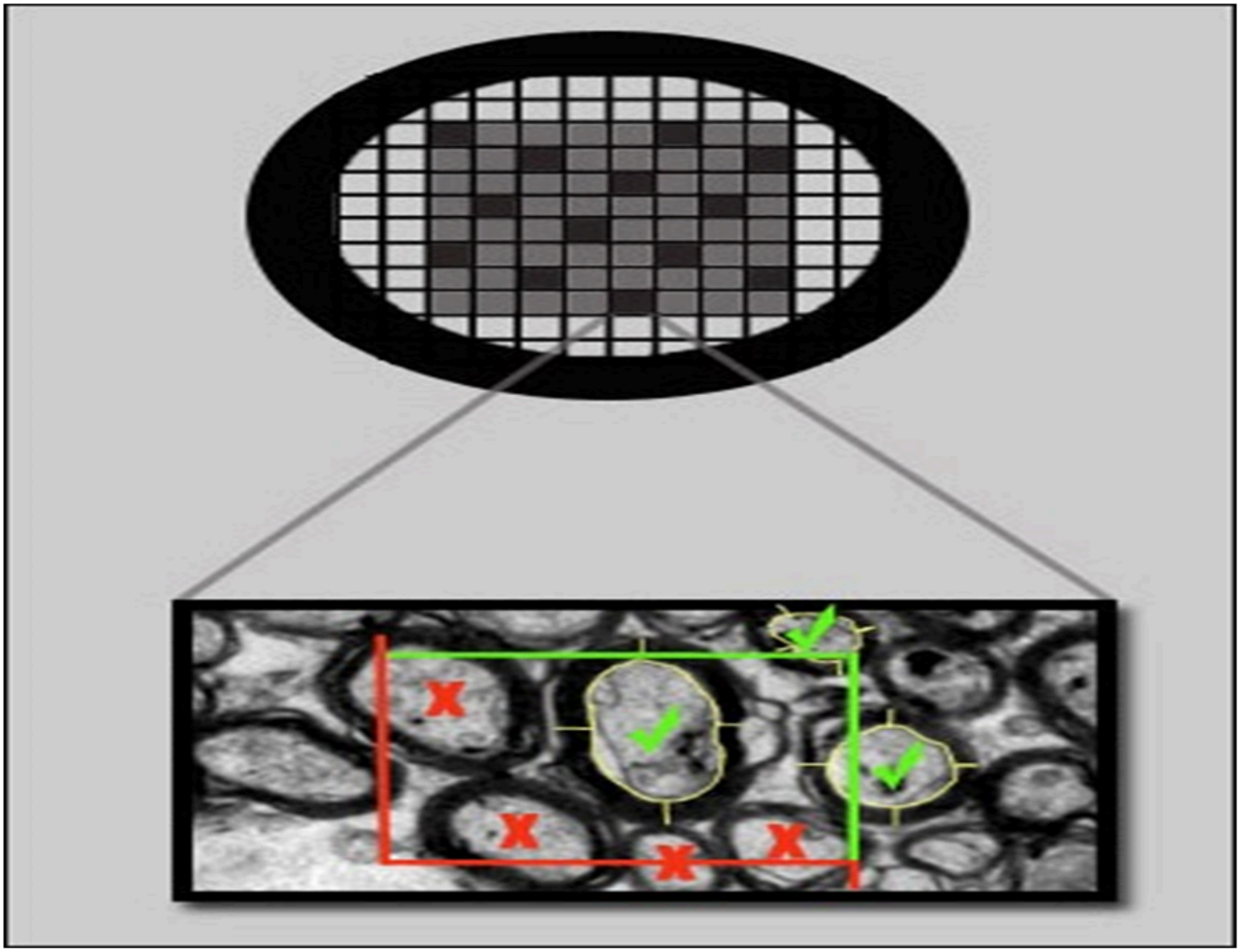


Figure 1

The light microscopic images represent the stereological analysis using Stereoinvestigator. Contouring the nerve fascicle at the x4 magnification (A) and counting the myelinated axon number at the x63 magnification (B) are shown. At the same time, the axon area and myelin sheath thickness at x63 magnification were estimated using the nucleator method (C).

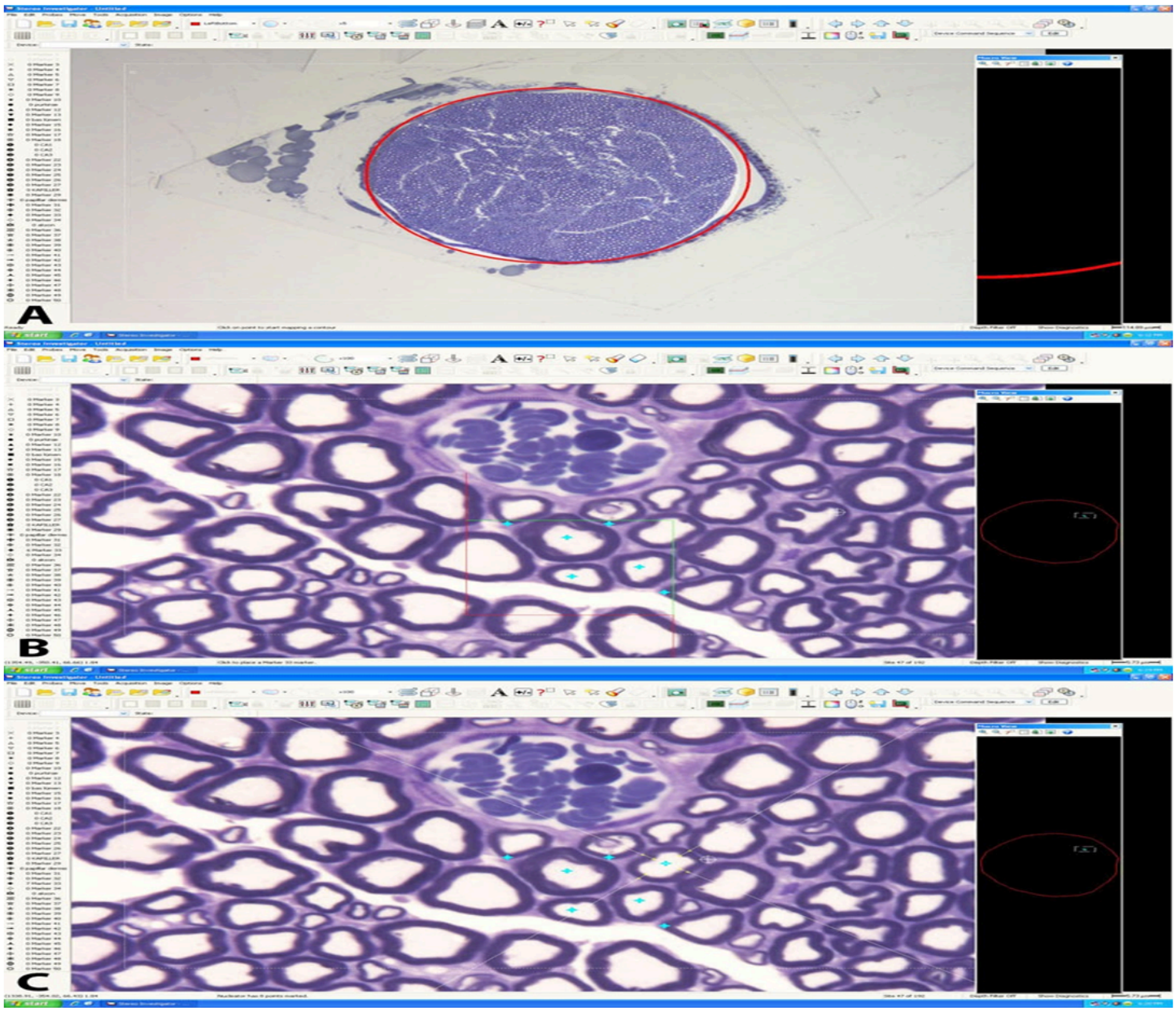


Figure 2

The schema represents the Fractionator method using systematic random sampling and an unbiased counting frame. Included axons are shown by (√), and excluded axons are shown by (X).

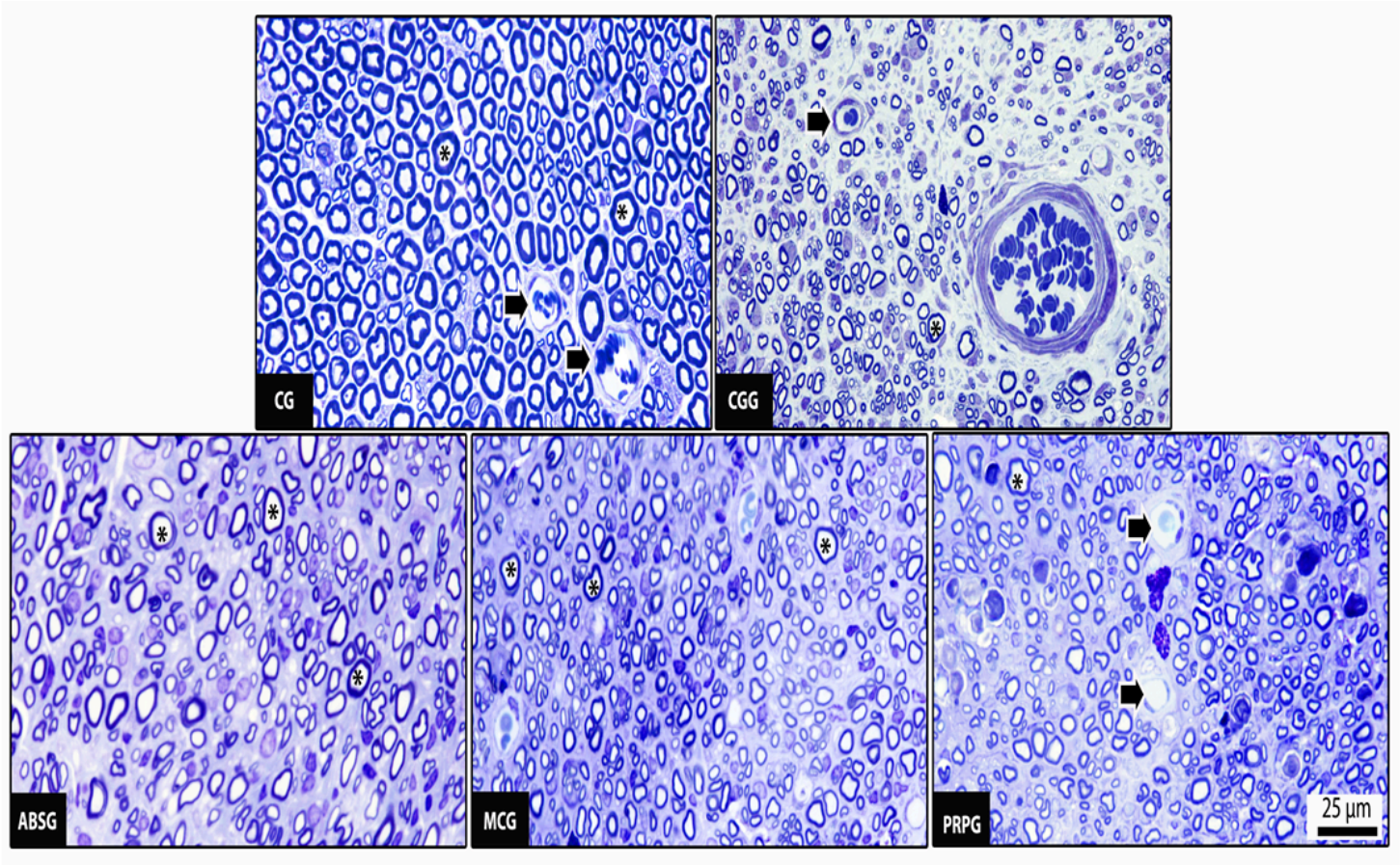


Figure 3

The light microscopic images are given for each group. The black arrows indicate the vessels, and also (*) indicates the myelinated axons.

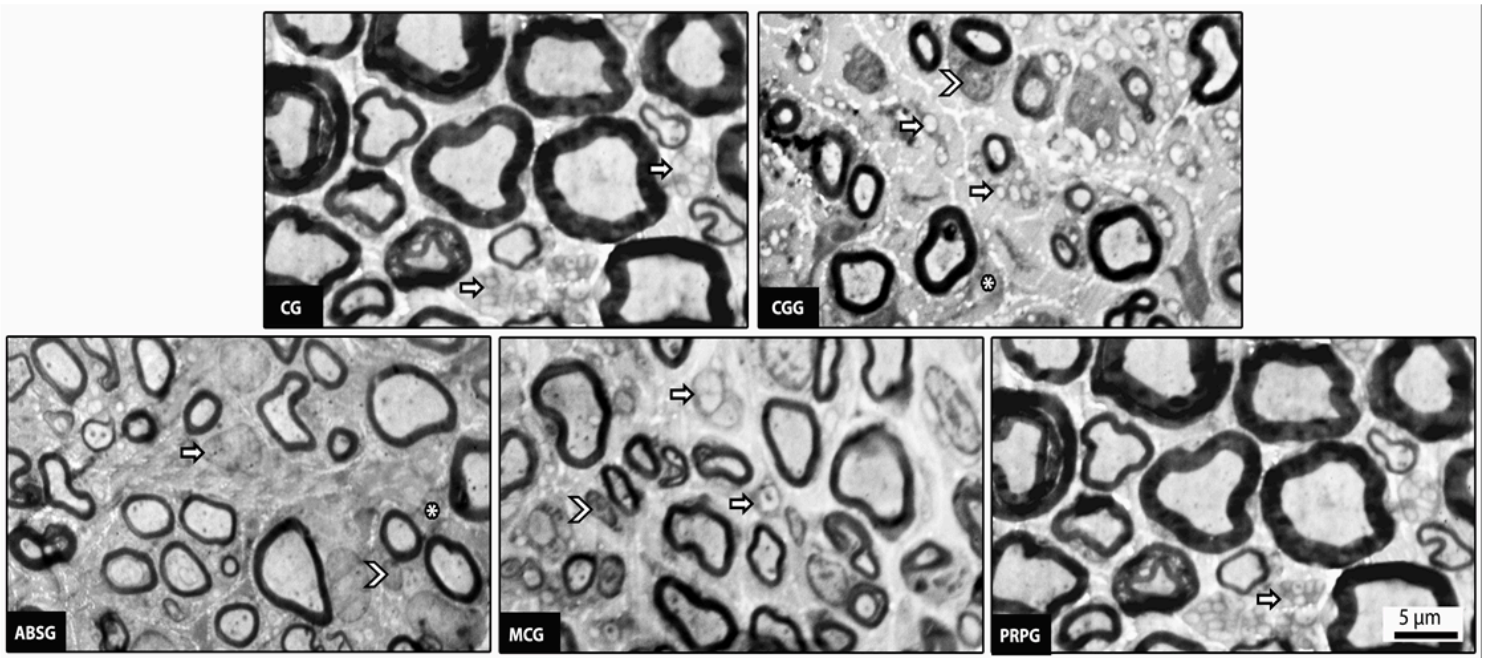


Figure 4

Transmission electron micrographs are given for each group. The white arrows indicate the unmyelinated axons, and (*) indicates the collagen fibers of the endoneurium. Additionally, Schwann cells are shown by white arrowheads.

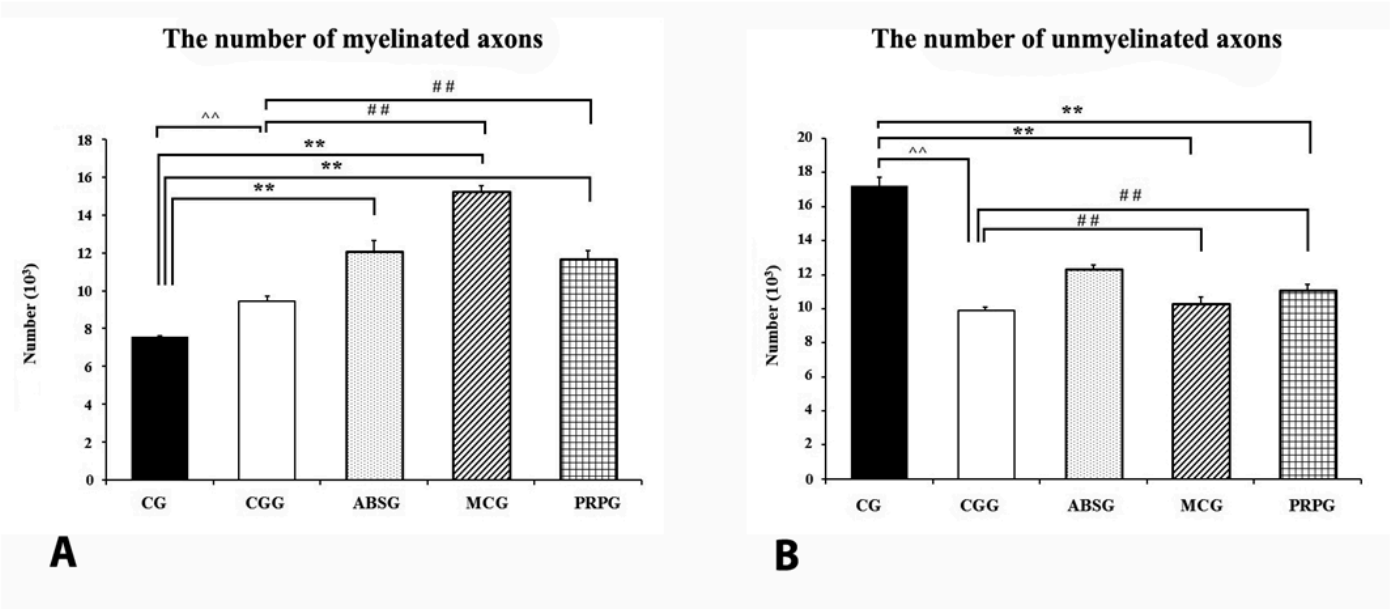


Figure 5

The graphical representation of the comparison among groups in terms of the total number of myelinated (A) and unmyelinated fibers (B) obtained in light and electron microscopes. Data are expressed as standard error mean. (**),(##), (^^) indicate the $p<0.01$.