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RESEARCH ARTICLE

Comparison of Fixation Methods for Peripheral Nerve Fiber [1] [2]

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Summary

Accurate fixation is a must for the assessment of myelinated nerve fiber morphology and transcardial perfusion and immersion methods are the most commonly used fixation techniques. In the present study we designed a new fixation technique for the histomorphometric and stereological evaluation of sciatic nerve fiber and referred it as instillation fixation. The method involved preliminary in situ fixation of the nerve sample without dissecting it from the animal body followed by a complete conventional fixation protocol. The objective of this study was to compare the three fixation techniques with each other for fixation artifacts. Eighteen female Wistar albino rats constituted the study material. The animals were allocated into three experimental groups corresponding to different fixation methods (immersion, instillation and transcardial perfusion, respectively). Quantitative assessments of nerve samples harvested from the animals of each group included the number of total myelinated axons, normal myelinated axons, alterations in myelin compaction, myelinated axons with irregular fiber shape, and with myelin loops and g ratio. Results revealed that normal myelinated axons were markedly lower in the immersion fixation group compared to those of instillation and transcardial perfusion. Moreover a significant decrease was noted with respect to alterations in myelin compaction in the instillation fixation group. In contrast, no significant difference was observed in myelin thickness and axon cross sectional area. In conclusion instillation fixation technique proved to be a valid and simple method for the assessment of peripheral nerve morphology for further analyses in a rat model.

Keywords: *Rat, Sciatic nerve, Fixation method*

Periferal Sinir Tespit Yöntemlerinin Karşılaştırılması

Özet

Doğru bir tespit işlemi miyelinli periferik sinirlerin morfolojik değerlendirmesi için ön koşuldur. Kalp perfüzyonu ve daldırma en yaygın kullanılan tespit yöntemlerindendir. Çalışmamızda bu metotlara ek olarak damlatma tekniği olarak adlandırılan yeni bir tespit metodu uygulandı. Sinir doku örneği canlı hayvan üzerinden çıkarılmadan ön tespit işlemine tabi tutulduktan sonra rutin tespit işlemleri tamamlandı ve üç tespit yönteminin tespit artefaktları açısından birbirleriyle histomorfometrik ve stereolojik olarak karşılaştırılması gerçekleştirildi. On sekiz adet dişi Wistar albino sıçan kullanıldı. Hayvanlar sırasıyla daldırma, damlatma ve kalp perfüzyonu olmak üzere üç eşit gruba ayrıldı. Her bir gruptaki hayvanlara ait siyatik sinir örneklerinin kantitatif değerlendirilmesi toplam miyelinli akson sayısı, normal miyelinli akson sayısı, miyelin kompaksiyonundaki değişimler içeren aksonların sayısı, düzensiz demet yapısı ile kıvrımlı miyelin halkaları içeren miyelinli aksonların sayısı ve g oranları hesaplanarak gerçekleştirildi. Elde edilen bulgulara göre daldırma tespit grubundaki normal miyelinli akson sayısında, damlatma ve kalp perfüzyonu grupları ile karşılaştırıldığında belirgin bir düşüş belirlendi. Bununla beraber damlatma tespit grubunda miyelin kompaksiyonunda değişimler içeren akson sayısında istatistiksel olarak anlamlı bir düşüş kaydedildi. Buna karşın miyelin kalınlığı ve ortalama akson alanında farklılık gözlenmedi. Sonuç olarak sıçan modelinde damlatma tespit tekniğinin periferik sinir morfolojisinin değerlendirilmesinde geçerli ve pratik bir metot olduğu gösterildi.

Anahtar sözcükler: *Sıçan, Siyatik sinir, Tespit metotları*

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INTRODUCTION

Studies concerning peripheral nerve injuries and diseases have been drawing growing attention among researchers with the goal of improving diagnosis and treatment. Assessment of peripheral nerve morphology or in other words the morphology of myelinated axons is the cardinal of the investigation of nerve damage and regeneration [1-3].

Histomorphometric analysis of peripheral nerves is an intriguing topic for researches. Such methods involving computer-based nerve histomorphometry provide relatively unbiased and accurate assessment of quantitative features of nerve fibers, such as myelinated axon number, axonal cross-sectional area and myelin thickness and abnormalities in myelin structure when compared with conventional qualitative/visual examination methods and have been widely utilized both to determine the morphological characterizations of uninjured nerve samples and to investigate the response of the nerve tissue following several types of pathological conditions including injuries or any kind of therapeutic and surgical approaches [2,4]. Accurate analysis, however, depends on high-quality fixation of peripheral nerve tissue. The objective of fixation is to preserve the tissue sample as close to its natural form as possible, though even subtle changes in the intrinsic structure of the tissue sample, inevitably give rise to artifacts [5].

Transcardial perfusion and immersion are the two most common methods used for fixation, the former being accepted as the gold standard for the majority of the studies of neural tissue although some researchers pointed out the non-essentiality of transcardial perfusion, which was considered to be replaced by immersion of nerve specimens in the fixative agent after being dissected [5].

In this study we designed a novel fixation technique, which was termed as instillation fixation in a rat model and investigated the accuracy of this method in terms of several quantitative features of nerve fibers such as total axon number, the number of axons with no morphological alterations (number of normal axons), mean cross sectional area of axon, myelin thickness, alterations of myelin compaction, number of axons with irregular shape and myelin loops in the axoplasm (infoldings) as well as g ratio.

MATERIAL and METHODS

Experimental Design

Eighteen female Wistar albino rats, weighing between 200-250 g, obtained from the Experimental Medical Research Institute of Istanbul University, Istanbul, Turkey, were used in the study. The experimental animal protocol was carried out at Kafkas University, Faculty of Veterinary Medicine, Kars, Turkey and the tissue specimens were submitted to our laboratory for the whole tissue processing and assessment methods. The experiment was approved by The Animal Experiments and Ethics Committee of Kafkas University (No: 2011-46).

Animals were randomized into three groups (n=6 per group) and subjected to different fixation techniques: group I. Immersion fixation, group II. Instillation fixation and group III. Transcardial perfusion.

Before any manipulation, animals were anesthetized by single intramuscular injection of ketamine HCl (Ketalar, 50 mg/ml, Pfizer-Istanbul).

An oblique gluteal skin incision and a muscle-splitting incision were used to expose the sciatic nerve. The procedures in all groups were performed on the right sciatic nerve and the nerve was dissected after the whole experiment was completed and then the rats were sacrificed.

For all fixation procedures 2.5% glutaraldehyde in 0.1M sodium cocadylate phosphate buffer (pH 7.4) was used.

Immersion group: After being dissected the nerve specimen was immersed in the fixative solution.

Instillation group: Prior to the dissection, sciatic nerve was prefixed in situ through being embedded in a pool of the fixation solution formed by slightly lifted surrounding muscle tissues for 5 min. Then the nerve specimen was dissected and immersed in the fixative solution to complete fixation.

Perfusion group: Unlike the above mentioned procedures the animals in this group were heparinized before being anesthetized. The chest wall was opened to expose the beating heart, and a needle is then inserted into the left ventricle with an incision concurrently made in the right atrium. Perfusion was initiated with 400 ml of 0.1M sodium cocadylate phosphate buffer until the liver has become pale, assuring that the blood was rinsed off and the procedure was sustained with 400ml of 2.5% gluteraldehyde as the fixative. Finally the sciatic nerve was dissected and immersed in the fixation solution.

Tissue Processing

Nerve specimens belonging to the animals in all three groups were kept in the fixation solution for 24 h in 4ºC. Following fixation, the tissues were rinsed three times in sodium cocadylate phosphate buffer (pH 7.4) for 10 min. Then the tissues were postfixed in 1% osmium tetraoxide for 2 h. The tissues were rinsed once more in sodium cocadylate phosphate buffer (pH 7.4) for 10 min. The specimens were then dehydrated for 10 min in each of the following solutions using the following concentrations of ethanol; 3x70%, 3x 80%, 3x 90% and 3x 100%, respectively. Then the specimens were treated three times for 10 min each with pure propylene oxide (Sigma) and placed overnight in a sealed bottle including 1:1 mixture of propylene oxide and resin (Epon embedding kit/ Fluka Chemie Gmbt, Switzerland). The following day, the bottles were unsealed and remained intact for 3-4 h allowing the solution to evaporate. Then the specimens were

treated with epoxy resin for 24 h at room temperature. The whole procedure was completed by embedding the tissues in Epon Embedding Kit for 48 h at 60°C. For embedding, we used a silicon embedding mold that has 21 consecutively numbered, bullet-shaped cavities with a depth of 5 mm each. Semi-thin and ultra-thin sections (of 1 mm and 90 nm thickness, respectively) were cut by an ultramicrotome (Super Nova Reichert-Yung,Austria) and stained with 1% toluidine blue (semi-thin sections) and uranyl acetate-lead citrate (for ultra-thin sections), respectively. Ultra-thin sections were analyzed using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View III digital camera and Soft-Imaging System (SIS, Munster, Germany) [6]. Semi-thin sections were evaluated by light micro-scopy.

Stereological Analysis

Stereological analyses of sciatic nerves were conducted according to principles described previously [3,7,8]. A stereological workstation composed of a digital camera (mbf/Bioscience, Qimaging), otomatic controlled specimen stage, a light microscope (Leica, DM400B) and a software program (mbf Bioscience, Stereo investigator, version 9) was used to count axons. To obtain an estimation of total myelinated axon number in an unbiased manner, the axon profiles in the nerve cross-section were sampled with equal probability regardless of shape, size, orientation and location, which meant that each sampled item was selected with a systematic random manner [6]. For this aim, we chose an area fraction approach with an area of unbiased counting frame of 900 mm^2. Meander sampling of each sectioned nerve profiles was done in 70 µm x70 µm step size in a systematic-random manner, as well, ensuring that all locations within a nerve cross-section were equally represented and that all axon profiles were sampled with an equal probability regardless of shape, size, orientation and location [6,9].

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 [10,11] was used for estimation of cross-sectional axon area and the thickness of myelin sheet using an oil objective (100x, NA 1.40). Meander sampling of each sectioned nerve profiles for axon cross-section area and myelin sheet thickness was done over successive, systemic-random steps of 70 µm-70 µm. Two dimensional nucleator at isotropic uniform random positions was used for estimation of axonal areas and the thickness of myelin sheet using an oil objective (100x, NA 1.40) [12].

After the tissue processing methods, all the nerve samples belonging to the animals in each group were histomorphometrically evaluated with the aid of a video monitor connected to the microscope at a final magnification of 100× NA 1.40 [13] according to the following parameters, which were previously obtained on the basis of stereological analysis: total axon number, the number of axons with no morphological alterations (number of normal axon/undamaged axon), mean cross sectional area of axon, myelin thickness, alterations of myelin compaction, number of axons with irregular shape and myelin loops in the axoplasm (infoldings) as well as g-ratio. G-ratio was calculated as the quotient between the axon perimeter and the myelin perimeter.

Statistical Analysis

At least six rats were studied for each experimental group. The "n" used for statistical analysis was the number of animals. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Duncan test using the SPSS 13.0 programs. Statistical significance was established as $P<0.05$.

RESULTS

Histomorphometric Evaluation

Several parameters were histomorphologically identified for the assessment of alterations in the size and shape of myelinated fibers corresponding to different fixation methods. Myelin invaginations in the axoplasm (infoldings), axons with irregular shapes, alterations in myelin compaction, as well as normal (undamaged axonal structures were recognized and evaluated on semi-thin sections by light microscopy. Electron micrographs of these abnormalities in myelin compaction revealed incisures and general lamellar separation of myelin sheath *(Fig. 1)*.

Stereological Analysis

Total and normal myelinated axon numbers: No statistically significant difference ($P>0.05$) was noted between the groups with respect to total myelinated axon number although a statistically significant difference was found in the immersion group in terms of normal myelinated axon number ($P<0.01$) *(Fig. 2)*.

Estimation of Myelin thickness and axonal cross section area: The groups were found to be identical in terms of mean cross sectional area of axon and thickness of myelin sheath, thus no significant difference was statistically detected in parallel with the histomorphometric evaluation ($P>0.05$) *(Fig. 3)*.

Alterations in myelin structure: The most frequent of these abnormalities was the presence of alterations in myelin compaction. A prominent decrease was detected in the instillation group with respect to the number of the axons with alterations in myelin compaction, which was statistically significant ($P<0.05$). However, no statistically significant difference was detected between the groups in terms of the number of axons with irregular shapes and myelin loops ($P>0.05$) *(Fig. 4)*. Percent values of abnormalities of myelin structure remained unchanged within the total myelinated axon number and these values were summarized in *Fig. 5*.

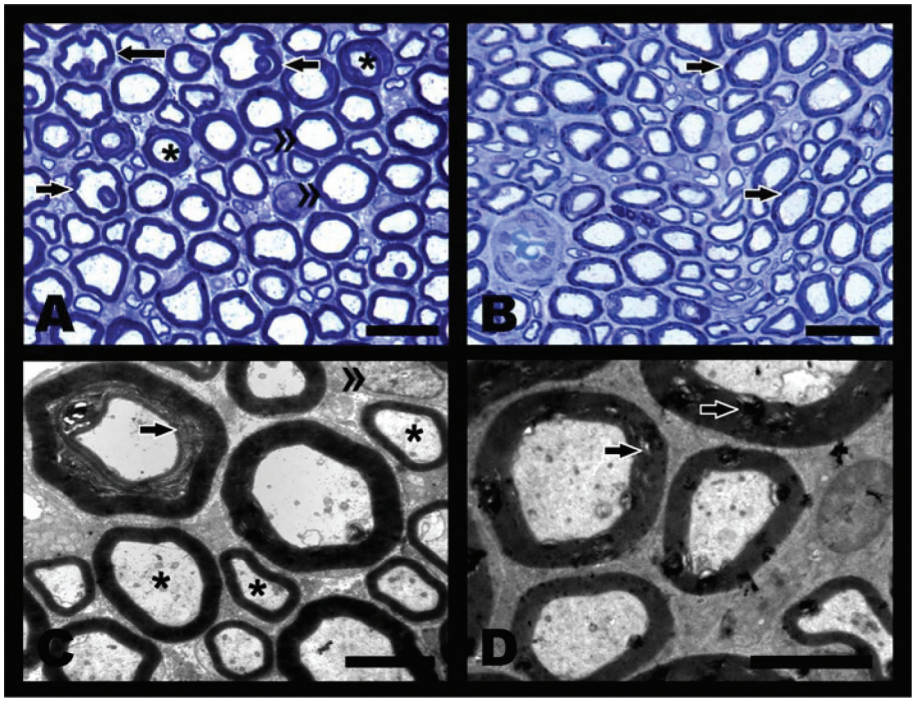

Fig 1. Light and electron microscopic views of sciatic nerve fibers. **A-** Recognition of myelin fiber abnormalities in the sciatic nerve. *Asterisks:* Alterations in myelin compaction (General lamellar separation). *Long arrow:* Irregular fiber shape. *Short arrows:* Myelinated fiber with a myelin loop (infoldings). *Arrowheads:* Normal myelinated axon **B-** Section from Immersion Group. *Arrows:* Alterations in myelin compaction. Semi-thin section. Toluidine blue Stain. Scale bar, 20 µm. **C-** Section from Instillation Group. *Asterisks:* Normal myelinated axons. *Arrows:* General lamellar separation. *Arrowhead:* Schwann cell nuclei **D-** Section from Immersion Group. *Arrows:* Incisures in myelin sheath. Ultra thin sections. Uranyl acetate-lead citrate stain. Scale bar, 5 µm

Şekil 1. Siyatik sinir demetlerinin ışık ve elektron mikroskobik görüntüleri

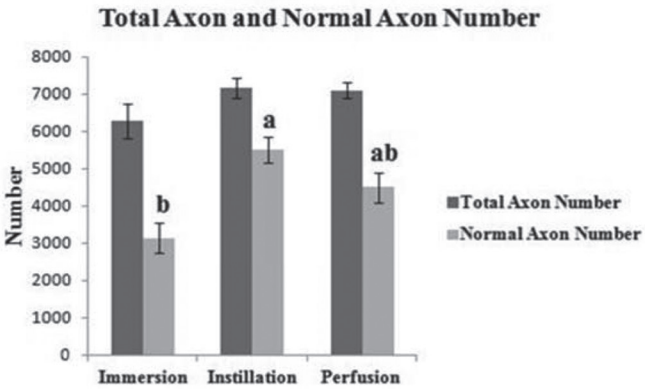

Fig 2. A comparison of the numbers of total myelinated axons and normal myelinated axons in the sciatic nerve. The graphic reveals a homogenous distribution of total axon number in all experimental groups (P>0.05). a, b, ab indicates the differences between the groups. ** P<0.01

Şekil 2. Siyatik sinirdeki toplam miyelinli akson ve normal miyelinli akson sayılarının karşılaştırılması

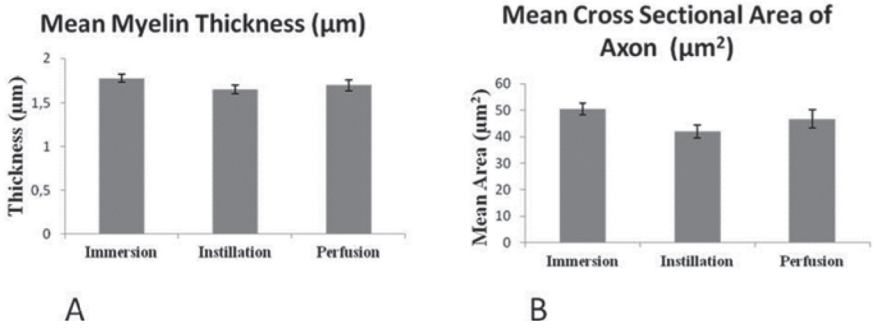

Fig 3. A comparison of the mean myelin thickness **(A)** and cross sectional area of axon in the sciatic nerve **(B)**. No statistically significant difference was noted between the groups (P>0.05)

Şekil 3. Siyatik sinirdeki miyelin kalınlığının **(A)** ve ortalama akson alanlarının **(B)** karşılaştırılması

Quantitative assessments of shrinkage for the three experimental groups were performed by Axon to myelin ratio (g-ratios). This ratio did not differ between groups, indicating no differential shrinkage was present *(Fig. 6)*.

DISCUSSION

Tissue fixation is the most essential step of all tissue processing protocols allowing further analyses. The goal of fixation is to preserve cells and tissue constituents in as close a life-like state as possible by preventing postmortem decay. However, the dilemma of fixation has always been that it introduces some artifact, which is observed as alterations of the original chemical and physical compositions of tissues [14,15]. A proper fixation protocol is crucial, as well for the studies on histomorphometric evaluation of peripheral nerve fibers particularly concerning the quantitative features such as axon number, axonal cross-sectional area and

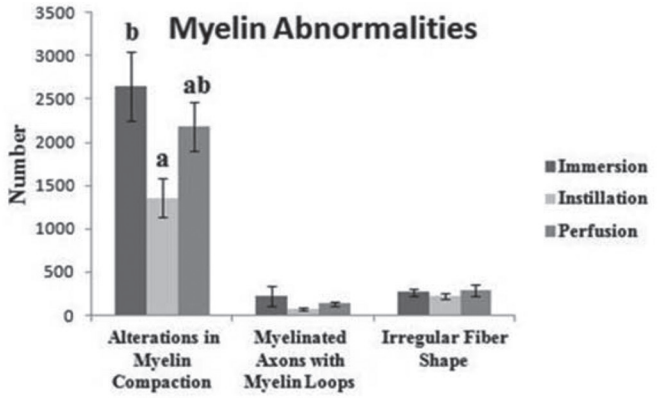

Fig 4. A comparison of myelin abnormalities corresponding to all three groups. a, b, ab indicates the differences between the groups in terms of alterations in myelin compaction * P<0.05 reveals immersion group. The number of myelinated axons with myelin loops and irregular fiber shape are non-significant (P>0.05)

Şekil 4. Tüm gruplar arasındaki miyelin anomalilerinin karşılaştırılması

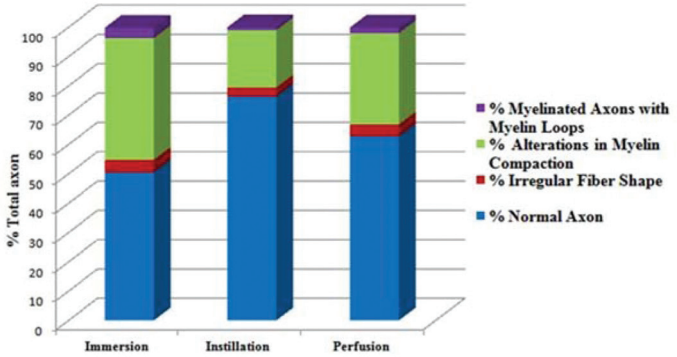

Fig 5. Percent values of myelin abnormalities within the total myelinated axon number

Şekil 5. Toplam miyelinli akson sayısı içindeki miyelin anomalilerinin yüzde değerleri

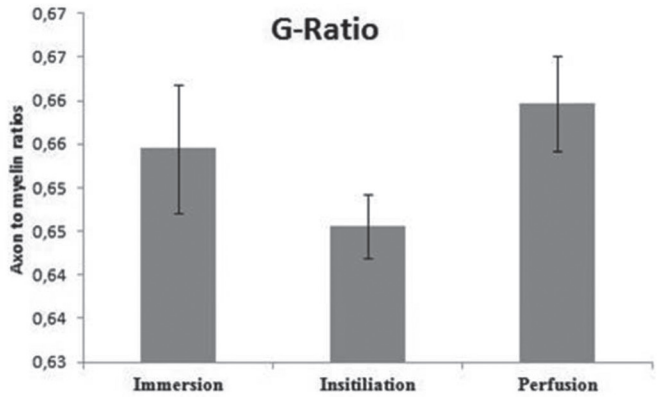

Fig 6. G-ratio (axon to myelin ratios)

Findings were similar across all groups, indicating that no preferential shrinkage artifact occurred with any of the fixation regimens (P>0.05)

Şekil 6. G-oranı

myelin thickness [2,4]. Most commonly used fixation protocols in these studies are immersion and perfusion fixation techniques [5,14,16]. Although transcardial perfusion has been approved as the most effective method it indeed has several disadvantages. It occupies plenty of time, labor and definitely should be performed by expertised staff. Biosafety of the procedure is subject to interrogation, as well, since large amount of fixatives including suspected carcinogens are applied [5,17]. A shortfall of transcardial perfusion is that the peripheral nerve can be curved by muscular contractions during the procedure and may thus be fixed in a curved shape, which makes sectioning tricky [1]. Immersion has lately been proved to be superior to conventional perfusion method due to its less time and labor intensiveness, practicality and cheapness. The staff is exposed to less volume of fixative with lesser time, as well [14]. It was considered to be more accurate in an aspect that structural distortions of the nerve samples due to muscular contractions during the transcardial perfusion method could be avoided in the immersion protocol since the nerve samples, immediately after having been dissected, were immersed in a small drop of fixative solution, kept in a straight position for a few minutes followed by immersion in fixative solution for complete fixation [15,18].

In this study we developed a novel technique, which was considered to be an alternative also to immersion and referred it as instillation fixation. Our experience of the studies we carried out with respect to peripheral nerve tissues revealed that metachromasie, observed as the pale staining of the nerve tissue samples, particularly in the middle sections was the handicap of the immersion method due to the insufficient diffusion of the fixative agent throughout the sample. In the immersion method, though superior to perfusion in above mentioned aspects, postmortem decay process was already initiated during the period the nerve was dissected

until immersed in the fixation solution, which took approximately 1.5-2 min and this was an *in vitro* process [5]. In the instillation fixation since the fixation process has already started while the animal was still alive, an adequate diffusion of the fixative through the entire sampled nerve was achieved, which eliminated the artifacts such as pale staining and therefore the quality of fixation was improved for further protocols. Another striking point was that no structural changes such as curves occurred in the sciatic nerve since the natural position of the nerve was preserved while the fixative solution properly penetrated into the tissue concurrently.

Consistent g-scores proved that no quantitative difference was found between the groups with respect to shrinkage and thus deceptive results were eliminated in terms of myelin thickness and axonal cross-sectional area.

No statistically significant difference was detected between the groups with respect to total axon number. However, normal axon number was found to be reduced in the immersion group. On the other hand, number of axons with alterations in myelin compaction significantly increased in the same group. This might be associated with the delayed and insufficient penetration of the fixative solution during the process, which resulted in artifacts as separation of lamellae of myelin sheaths. The mechanic pressure upon the inadequately fixed tissue samples during the sectioning might have caused separation of myelin sheaths. The electron microscopy data served to further demonstrate the flaws of the immersion technique. This difference might be associated with the individual diversity of each animal with respect to total myelinated axon number in the sciatic nerve. However the statistical insignificance of this parameter between the groups and identical percent values for alterations in myelin structure in all groups ruled out this conception. On the contrary, perfusion and instillation groups were equivalent in terms of all parameters investigated due to the constancy of the percent values of the parameters concerning the alterations in myelin structure within the number of total axons. In the previous studies [1,5] immersion technique was introduced as an alternative and in some aspects superior to perfusion method. However, our results pointed out the inadequacy of immersion technique parti-cularly demonstrated as a prominent increase of alterations in myelin compaction and a decrease of number of normal axons. Other myelin abnormalities such as irregular arrangement of myelin sheaths, formation of loops between layers and infoldings into the axoplasm reflected no significant differences among the groups since these parameters were known to be observed also with aging without any pathological condition [19,20] regardless of the method of fixation.

In conclusion, our data indicated that instillation fixation technique is capable of reducing morphological abnormalities of myelin in the sciatic nerve. Therefore, this technique was considered to be a practical method allowing accurate assessment of peripheral nerve morphology in a rat model.

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