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Research Article

Comparison of Lyophylized Duramater and Autogenous Omental Wrappings of Grafting Sites in Experimentally Induced Facial Nerve Injury*

Part II Histologic and Histomorphometric Evaluation

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Abstract: The histologic and histomorphometric evaluation of wrapping the facial nerve by a lyophylized duramater (LD) or autogenous omental graft (AOG), on nerve regeneration, after experimentally induced facial paralysis, which was created by neurectomy and repaired by autogeneous sural nerve grafts in dogs, was undertaken. The experimental design was reported. At the end of the observation period, specimens harvested from the main trunk of the facial nerve arising from the stylomastoid foramen, the repaired site and three branches of this nerve were examined histologically and histomorphometrically with respect to myelin area, axon number and nerve fiber diameter. Reduced fibrosis and improved nerve regeneration were found to be superior in the AOG wrapped group than the others. According to the histomorphometric evaluation of grafted site, the wrapped groups had better values than those of the control group. Wrapping was found to be useful for nerve regeneration in the grafting site and the omentum could be recommended for this purpose. Some reference values were obtained from histomorphometric evaluations that will be helpful for further studies, and traumatic injuries of facial nerves can be repaired by free cable grafts and regeneration seemed better when wrapped with AOG.

Key Words: Duramater, facial paralysis, graft, nerve regeneration, omentum

Deneysel Fasial Sinir Yaralanmalarında Greft Uygulanan Bölgenin Liyofilize Duramater ve Otojen Omentum ile Sarılmasının Karşılaştırılması:

İkinci Bölüm: Histolojik ve Histomorfometrik Değerlendirme

Özet: Bu çalışmada, fasial sinirin nörektomisi ile oluşturulan deneysel fasial paralizinin otojen sural sinir greftleri ile onarılmasından sonra fasial sinirin liyofilize duramater ve otojen omentum grefti ile sarılmasının sinir rejenerasyonuna etkisi histolojik ve histomorfometrik yönden değerlendirilmesi amaçlandı. Deneysel çalışma planı birinci bölümde bildirildi. Gözlem süresi sonunda, fasial sinir foramen stilomastoideustan çıktıktan sonraki ana trunkus, onarım bölgesi ve fasial sinirin üç kolundan alınan kesitler miyelin alanı, akson sayısı, sinir lifi çapı yönünden histomorfometrik ve histolojik olarak incelendi. Histolojik ve histomorfometrik analizlere göre otojen omentum grefti ile sarılan grupta fibrozisin diğer gruplara oranla daha az, sinir rejenerasyonunun ise daha iyi olduğu belirlendi. Greft alanında yapılan histomorfometrik incelemeler sonucu, sarma işlemi yapılan gruplarda kontrol grubuna oranla daha iyi değerler elde edildi. Greft bölgesinin sarılmasının, sinir rejenerasyonuna yararlı olduğu saptandı ve bu amaç için otojen omentumun önerilebileceği sonucuna varıldı. Çalışmada daha sonraki araştırmalara ışık tutacak bazı histomorfometrik referans değerler elde edildi ve travmatik fasial sinir yaralanmalarının kablo greftleri ile onarılabileceği ve otojen omentum grefti ile yapılan sarma işleminin sinir rejenerasyonuna katkıda bulunabileceği kanısına varıldı.

Anahtar Sözcükler: Duramater, fasial paralizi, greft, sinir rejenerasyonu, omentum

Introduction

Facial paralysis is well documented in dogs and cats, especially with regards its etyiology and diagnosis. The most common cause of facial neuropathy was judged to be idiopathic, but traumatic facial nerve injury is not rare in small animal practice (1,2). If circumstances are suitable in humans, facial paralysis can be treated properly with direct repair or grafting (3-5). The regenerated nerve is observed by clinical, histomorphometric and electrophysiological evaluations. In the histomorphometric evaluations, myelin sheath thickness, nerve fiber diamater and axon counts are taken into account by many authors (6-8).

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The present paper describes histomorphometric aspects of the role of wrapping the grafted site by the LD and AOG with respect to nerve fiber diameter, myelin sheath thickness, and axon counts. Histologic examination of the repaired site was also evaluated. It was considered that the histologic and histomorphometric evaluations of the facial nerve of the adult dog would determine which wrapping material was suitable.

Materials and Methods

Case identification, experiment design, operative technique and observation period were reported previously (9).

The specimens were harvested from the grafted site, the three main branches (auriculopalpebral, dorsal buccal and ventral buccal branch) and the main trunk arising from stylomastoide foramen of the healthy side. All specimens were fixated separately in 2.5% gluteraldehyde solutions. The tissues were washed with caccodylate sodium in 0.1 molarity and they were fixed subsequently for 2 h in osmium tetroxide solution. The specimens were rewashed with cacodylate sodium (0.1 molarity) and then dehydrated in ethanol solutions (30, 50, 70, 90 and 100º). The specimens were kept in araldite (araldite CY 212 + araldite HY 964 + araldite DY 064 + Dibutyl phthalate) and propylene oxide mixture for 12 h. They were embedded in araldite. The prepared blocks were hardened at 40ºC for 24 h and 60ºC for 24 h consecutively. Sections of 1 µm thickness were cut using a 8800 LKB ultramicrotome.

Histomorphometric evaluation of the sections stained with Toluidine Blue was performed by a video camera connected to a light microscope and by a computer (Leica Q 500) having an Image Analyzing Program (KS400 Software). Myelin sheath thickness and nerve fiber diameter were calculated as the inner and outer border of myelin sheaths. They were randomly selected from 50 different areas (x100 magnification) and were drawn manually and calculated by a computer. Myelinated axon counts were estimated in at least 5 different areas of each section. Axons that appeared to be rectangular or square in shape were not estimated.

The outer diameter of a myelinated axon was taken into account to determine the nerve fiber diameter. The average value of each lesser and greater diameter was calculated by the computer to estimate nerve fiber diameter. Nerve materials, taken from grafted sites, were stained via the Klüver Barrera method (10).

The results were analyzed with the Chi-square test.

Results

Histomorphometric Analysis: The values of axon counts, nerve fiber diameter and myelin sheath thickness estimated from semi-thin sections of the main facial nerve trunk, the grafted site and the three branches are introduced in Figures 1-3.

Average axonal number was 5116.6, mean nerve fiber diameter was 18.19± 9.32 µm and myelin sheath thickness was 43.25 μm^2 in the main facial nerve trunk.

There were no significant differences between the groups on axon counts. Although significant difference was not present between the branches ($P > 0.05$), axon counts in the grafted sites were less than each of three branches (Figure 1). Nerve fiber diameters of the main trunk were greater than the grafted site and all three branches but the AOG group had a greater value than the others ($P>0.05$) (Figure 2).

A significant difference of myelin sheath thickness was not detected between the branches of each groups, but it was greater in the AOG group ($P<0.05$) (Figure 3). It was smaller in the grafting site than in the branches, but it was insignificant. Myelin sheath thickness was greater at the sections of the main trunk than in both the grafting site and the branches. In the LD group, myelin sheath thickness was smaller than the others ($P<0.05$).

The means of nerve fiber diameter and myelin sheath thickness were superior in the AOG , control and LD groups respectively, even though it was not significant ($P>0.05$).

Histologic Evaluations: Periepineural and interfascicular fibrosis with slight cellular reaction including macrophage and hystiocytes were commonly seen in the two dogs of the control group. Vacuolar and

$$\text{Total axon counts} = \frac{\text{Transected nerve area}}{\text{Evaluated area}} \text{ X Myelinated nerve numbers in evaluated area}$$

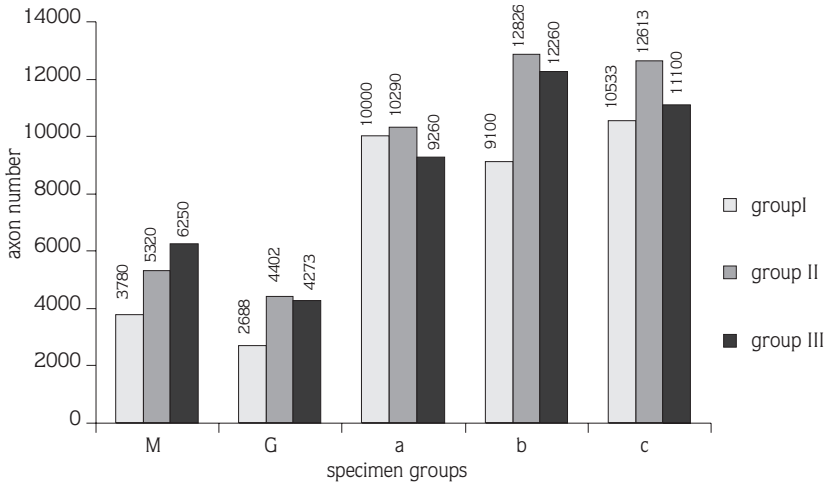

Figure 1. Axon Numbers of Each Group for the Three Branches, Grafted Site, and Main Truncus of Facial Nerve. M: Main Truncus of Facial Nerve, G: Grafted Site, a: Auriclopalpebral Branch of Facial Nerve, b: Dorsal Buccal Branch of Facial Nerve, c: Ventral Buccal Branch of Facial Nerve.

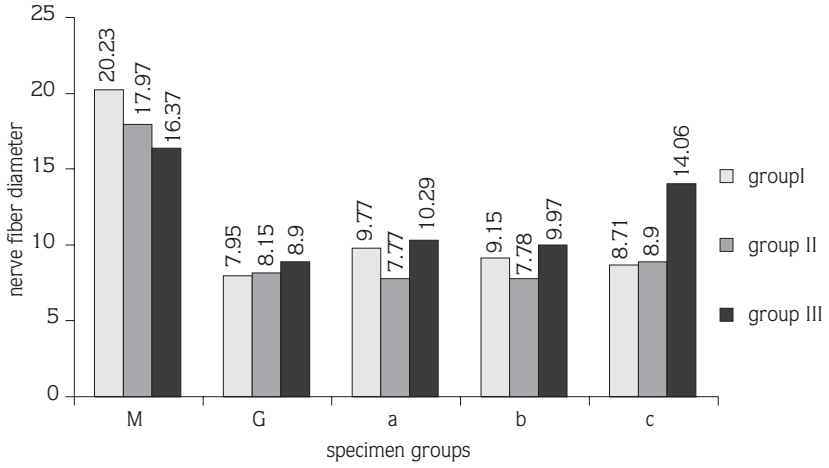

Figure 2. Nerve Fiber Diameter of Each Group for the Three Branches, Grafted Site, and Main Truncus of Facial Nerve. M: Main Truncus of Facial Nerve, G: Grafted Site, a: Auriclopalpebral Branch of Facial Nerve, b: Dorsal Buccal Branch of Facial Nerve, c: Ventral Buccal Branch of Facial Nerve.

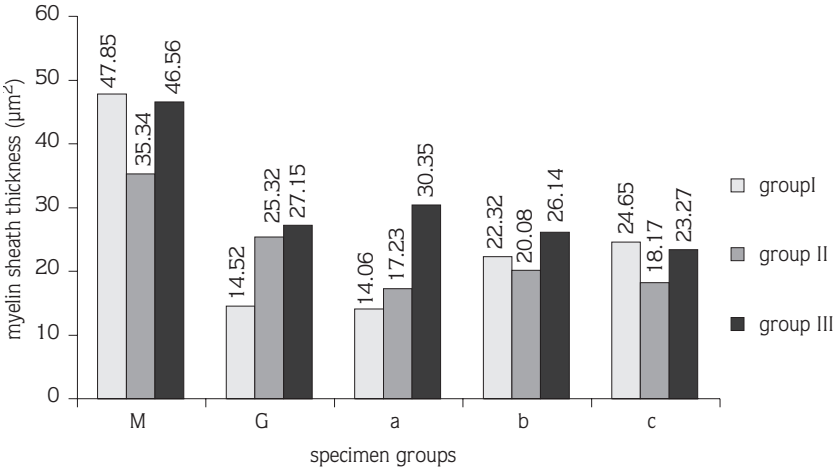

Figure 3. Myeline Sheath Thickness of Each Group for the Three Branches, Grafted Site, and Main Truncus of Facial Nerve. M: Main Truncus of Facial Nerve, G: Grafted Site, a: Auriclopalpebral Branch of Facial Nerve, b: Dorsal Buccal Branch of Facial Nerve, c: Ventral Buccal Branch of Facial Nerve.

granular structures were observed at the myelin layer in almost all nerve fibers and axonal necrosis (in one of these dogs). Free nerve fibers were seen extrafasiculary and were noted as irregular situations (Figure 4).

In the LD group, the repaired site was surrounded with slight fibrosis that contained multifocal inflammatory infiltration containing mostly lymphocytes and macrophages (Figure 5). Slight interfascicular

fibrosis, large numbers of free nerve fibers and neuroma formation were found in one dog of the LD group. Well myelinated axons and incomplete regeneration of the endoneurial tube in fascicular pattern were seen in the histologic section of the LD group (Figure 6). Foreign giant cell bodies and macrophages were detected around the suture material used for wrapping.

In the AOG group, there was fibrosis around the repaired and interfascicular region. However, both cellular reactions or fibrosis were lesser than in the other groups. Necrosis of some axons and Schwann cell proliferation were observed in one dog. A granular appearance was detected in the myelin layers of two dogs. Axons were well myelinated in different diamaters but not in regular fascicular pattern in the histologic section of the AOG group (Figure 7).

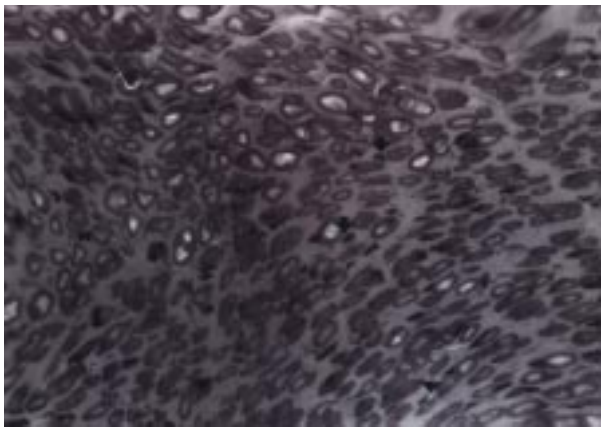

Figure 4. Incomplete myelin regeneration and irregular localized axons with many free schwon cell (Arrow) and degenerated axons with vacoullar structure (Arrow head) control group, semi-thin section, Toluidine blue x740.

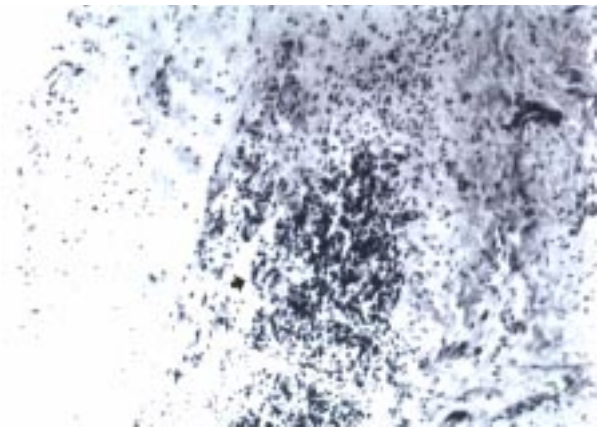

Figure 5. Macrophage and lympocyt infiltration between fibrous tissue and LD at the grafted site. LD group, Kluver Barrea x40.

Discussion

The nerve fiber diameter, myelin sheath thickness and axon counts, which are consider by many authors as a criterion in histomorphometric analysis (6,11,12) were used in the present study. To the authors' knowledge, there have not been any histomorphometric studies about facial nerve grafting in dogs. The facial nerve fiber diameter of a healthy adult dog is reported to be 3.92 ±1.18 µm. In the same study, approximately 89% of the facial nerve fiber diameters were between 3 and 6 µm and ranged from 2 to 12 µm (10). In the present study, mean nerve fiber diameter was estimated to be 18.19 ± 9.32 µm. In the study mentioned above (10), the nerve fibers of a circular shape and the diameters of lesser axis were taken into account, crenate fibers were not measured. In our study, nerve fibers every shape were

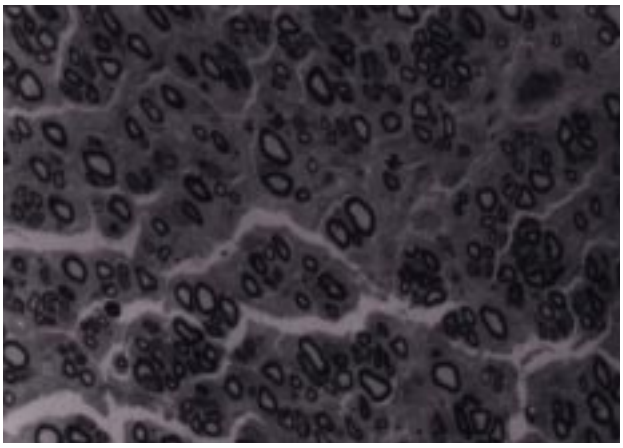

Figure 6. Moderate myelinised and different size regenerated axons (arrow), notice the fasicular structure of the axons, LD group, semi-thin section, Toluidine blue x740.

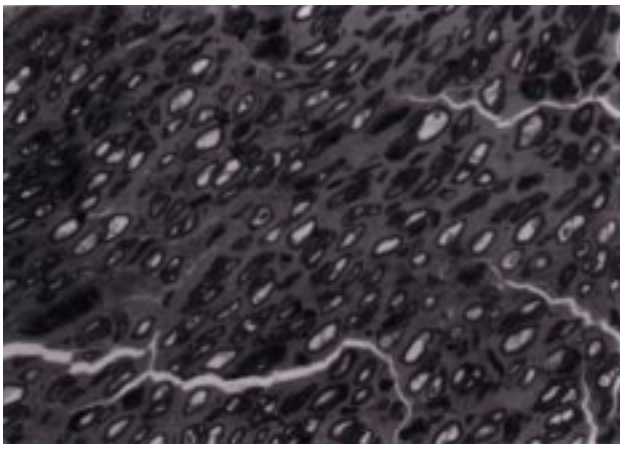

Figure 7. Well myelinised and regenerated axons in homogenous size AOG group, semithin section, Toluidine blue x740.

estimated and the average value of lesser and greater diameter fibers were evaluated. The cause of the disagreement with the other study (10) was a result of different evaluation methods.

According to previous studies, axon counts are not enough to evaluate peripheral nerve regeneration, despite its extensive use for this purpose (4,11,12). Regenerated axons can branch into as many as 20 distal fibers, and axon counts in the distal segment may be 150% greater than that in the proximal segment (11). In the present study, axon counts were lower in distal parts of the grafted site than the proximal in all of the groups as seen in Figure 1. Not only were axon counts in the grafted site lower than axons of total three branches, but also it was less than the numbers of each one of the branches. It was confirmed that, even though few axons pass through the graft, they might be branched at the distal part of the branches because of the signs of clinical improvement. There were no significant differences between the groups according to axon counts ($P > 0.05$). As the three main branches of the healthy side, the results could not be compared to the normal number of axons. At the same time, there were no reference values about axon counts for branches of canine facial nerves to the authors' knowledge.

The relationship between nerve conduction velocity and the diameter of myelinated nerves are known. The fastest conduction is seen in the largest diameter nerve fiber. Nerve fiber diameters are greater in the proximal part of the grafted site than in both the grafted site (3) and the distal part of it in the peripheral nerve of extremity (11,12). Thanos and Terzis studied cross facial nerve grafting on the basis of axon number, and nerve fiber diameter and myelin sheath thickness (8). They observed that the value detected in the proximal part of the grafted site was the greatest, and the value in the grafted site and distal part of the grafted site were smaller. In a study on grafting facial nerve after neurectomy, axonal diameter and myelin area in the grafted site were reported to be smaller than the distal part of the grafted site (13). The results of the study showed that sections taken from the facial nerve trunk had a greater diameter of nerve fiber than in the grafted site or the branches. Mean fiber diameter in the AOG group was greater than in the others.

Functionally, the myelin sheath serves as an insulator controlling the leakage of current. Its area would have to increase accordingly with nerve fiber diameter. Myelinization at the regeneration phase began when the growing axon reached the distal tube and were increasing from proximal to distal over time. This is a very important criterion for the establishment of regeneration (11,14). It is reported that regenerated and myelinated nerve fibers have thinner sheaths than normal (6). Myelin sheath thickness had a statistically smaller value in the grafted site and branches of the nerves in the LD group than in the others. It was observed that the appearance of myelin sheath thickness was not normal in all three groups. However, they appeared to be better in the AOG group than the others. The histomorphometric value of the three branches of the nerve fiber diameter, and the myelin sheath thickness of the LD group was lower than in the control group but it was thought that the results of analysis was responsible for this. During estimation, Schwann cell and vacuolar structure had to be taken into acount and affected the results. In histologic evaluation, dispersion of the well myelinated axons was less in the control group than in the wrapping groups. Extensive degenerated axons and Schwann cell proliferation was seen in the histologic sections of the control group.

Macrophage and fibroblast infiltration and adhesions were observed upon histologic examination of the repaired nerve. At neuroma formation, nerve fibers are dispersed in fibrous tissue randomly (11). Adhesion of repaired nerve to peripheral tissues, and fibrous tissue that was localized at both the interfascicular area and around the nerve in the histologic sections, was thought to be the result of unwrapping the repaired site. The results revealed the requirement of wrapping the grafted site to prevent fibrous tissue invasion into the repaired site. Well myelinated nerve fibers in an organized fascicular pattern were superior in the AOG, LD and control group concurrently. The superiority of the AOG group was as a result of its structural property, which not only protected the grafted site from peripheral invasion of the fibrosis, but also could affect the nutritional support of the graft.

Fibrous tissue formation was lower in the wrapped groups than in the controls and interfascicular fibrosis was limited at the LD wrapped area. It was evaluated that suture material caused foreign giant cell body formation and an inflammatory reaction which were closerued on the suture line. Lymphocyte invasion around the case of the LD group was interpreted as a minimum

antigenic property and the xenograft of LD. Minimum antigenic property and the gradual development of fibrous tissue instead of LD, which acts as a natural tissue, are the advantages of the LD (13). Because of the union of LD with fibrous tissue, it could not be recognized in some sections. In the AOG group, the omentum was united with fibrous tissue, and fibrosis was lower than in other groups. It was believed that the omentum reduced fibrosis. Observed fat tissue residue at the operated site was thought to be the omental structure. Neurons exiting from the suture line might be the reason for excessive neuroma formation in the control group. Axons exiting from the suture line inclined towards the distal direction by the guide of wrapping material were considered to be the reason for little or no neuroma formation in the cases wrapped with AOG or LD. Natural autogene tissue and the easy to fold properties of omentum might be the reason for lower neuroma formation than in the other groups. Excessive interfasciculary fibrosis in the control group might be the result of an invasion of fibrosis that came from around the repaired site.

In conclusion, injury of facial nerves can be repaired by sural nerve graft, but wrapping the grafted site should be considered with a suitable material. The results of the previous and present study indicate that AOG could be preferred for this purpose. There was no significant difference in three parameters between the branches of facial nerve. It was observed that almost equal regeneration was present in all the three branches.

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