

Effects of Parenteral Nutritional Support with Fish-Oil Emulsion on Spinal Cord Recovery in Rats with Traumatic Spinal Cord Injury

Traumatik Spinal Kord Yaralanması Olan Sıçanlarda Paranteral Balık Yağı Emülsiyonu ile Yapılan Beslenme Desteğinin Omuriliğin İyileşmesi Üzerine Etkileri

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ABSTRACT

AIM: Aim of this study is to assess effects of parenteral nutritional support with fish-oil emulsion on spinal cord recovery in rats with traumatic spinal cord injury.

MATERIAL and METHODS: For 5 days after SCI rats were received saline in group C and Omegaven in group O. Locomotor strengths (BBB scale) of animals were rated at Day 0,7,14,21,28, and 35. At Day 35 spinal cord sampling was evaluated immunohistochemically.

RESULTS: BBB scores were 0 in early period after SCI was inflicted in both groups. BBB scores were progressively increased after Day 7 in both groups ($p<.005$). BBB scores were significantly higher in group O when compared with control group after Day7 in all times ($p<.005$). Neuronal injury ($p<.002$) and edema was much more in control group when compared with in group O ($p<.005$). Scores for white mater cavitation, demyelination and vessel in growth were similar in both groups. VEGF expression in control group was higher ($p=.019$).

CONCLUSION: At the early period of SCI fish-oil emulsion treatment in rats, its anti-inflammatory effects leded to decrease in edema and had positive effect at the prevention of neuronal injury. We believe that nutritional support with fish-oil emulsion in patients with SCI will result in patient's better clinical outcome and increase in quality of the patient's life.

KEYWORDS: Omega-3 fatty acids, Omega-6 fatty acids, Neuron recovery, Nutrition, Spinal cord injury

ÖZ

AMAÇ: Travmatik omurilik yaralanması olan sıçanlarda omegaven ile beslenme desteğinin iyileşme üzerine etkilerinin araştırılmasıdır.

YÖNTEM ve GEREÇ: Omurilik hasarını takiben 5 gün boyunca sıçanlara IP 1mL/kg omegaven veya 0.9NaCl verildi. Lokomotor kuvvet (BBB skoru)0,7,14,21,28 ve 35.günlerde kaydedildi. Otuzbeşinci günde omurilikte histopatolojik inceleme yapıldı.

BULGULAR: Kontrol grubunda BBB skorları düşük ($p<.005$), nöronal hasarlanma ($p<.002$) ve ödem ($p<.005$) yüksek bulundu. Beyaz maddede kavitasyon, demiyelinizasyon ve damarlanma skorlarında fark saptanmadı. VEGF kontrol grubunda yüksek bulundu($p=.019$).

SONUÇ: Erken dönemde omurilik hasarında balık yağı emülsiyonu ile beslenme desteğinin antiinflamatuvar etki ile ödemi azalttığı ve nöronal hasar üzerine koruyucu etkisi olduğu görüldü. Omurilik yaralanması olan hastalarda balık yağı emülsiyonu ile beslenme desteği daha iyi klinik sonuçlar ve daha yüksek yaşam kalitesi sağlayabilir.

ANAHTAR SÖZCÜKLER: Omega-3 yağ asitleri, Omega-6 yağ asitleri, Nöron iyileşmesi, Beslenme, Omurilik hasarı

INTRODUCTION

Many people have spinal cord injury (SCI) by the minute worldwide, may it be as a result of car crash, motorcycle injuries, fall from height or athletic accidents, etc. (15). Traumatic injuries of the spinal cord lead to death of neuronal and glial cells and to degeneration of the ascending and descending tracts. It is generally considered that there are two main phases in the evolution of SCI; these are the primary injury at the site of impact which is inevitable and the secondary injury which reflects the gradual propagation of damage over a larger area of tissue. The pathogenesis of secondary injury includes excitatory toxicity, inflammation and increased oxidative stress. These events amplify the effect of the primary injury and continue in the days and weeks following the initial insult (9).

Mostly young patients, between 16 and 30 years old have SCI (15). The life expectancy for these patients is long and the patient's quality of life and cost of their neurological disorders depend on the prevention or treatment of secondary injury that occurs after primary SCI.

The inflammatory reaction in the acutely injured cord is an essential host defense mechanism, which functions to eliminate invading pathogens and clear debris. Inflammatory cells also promote wound healing events that support recovery. These beneficial events in both the acutely injured spinal cord and during wound healing may be overshadowed by an excessive accumulation of toxic molecules produced by inflammatory cells that damage otherwise intact tissue. As such, inflammation in the injured spinal cord has been regarded as a "two-edged sword" (3).

The central nervous system (CNS) is highly enriched in long-chain polyunsaturated fatty acids (PUFA) of the omega-6 and omega-3 series. Omega-3 and omega-6 PUFAs are components of membrane phospholipids, and have a structural role in all tissues, including the CNS (6). Besides, they have an anti-inflammatory effect (8). In particular, we were interested to know if including parenteral fish-oil emulsion in patients' nutrition protocol affects the recovery and rehabilitation of these patients with SCI.

The aim of this animal-SCI model study was to assess the effects of parenteral nutritional support with fish-oil emulsion (Omegaven®) on spinal cord recovery in rats with traumatic spinal cord injury, both as regards clinical outcome and immunohistopathological analysis.

MATERIAL and METHODS

The experimental protocol was approved by the Animal Care and Use Committee at Marmara University. Eighteen male Sprague-Dawley rats weighing approximately 250-300 g were housed with one animal per cage at Marmara University Institute of Neurological Sciences. The animals were fed a standard rodent chow diet and water ad libitum and kept at a constant temperature (22 °C) on 12-h cycles of light and dark.

The rats were anesthetized with an intra peritoneal injection of ketamine 90 mg/kg and then placed on an operating board in prone position. In each rat, the dorsal hair was closely shaved with an electrical razor, and the surgical field was disinfected with povidone-iodine and draped with sterile towels. A dorsal midline incision between the 5th and 10th thoracic vertebrae was performed. After paravertebral dissection of the muscle, laminectomy was performed on the 9th thoracic vertebra. The rats were randomly allocated into two groups (nine rats in each group) by using sealed envelopes, selected by a physician.

Group C (saline only control group; n=9): SCI was inflicted with Allen's spinal cord trauma method (see Allen's spinal cord trauma method below) (1). Each rat received a daily intraperitoneal (IP) injection of 1mL/kg physiological saline and 5mL/kg lactated ringer for 5 days.

Group O (Fish-oil only; n=9): SCI was inflicted with Allen's spinal cord trauma method. Each rat received a daily intraperitoneal (IP) injection of 1mL/kg fish-oil (Omegaven: Fresenius Kabi Austria GmbH, Graz, Austria; see formula for IV product below) and 5mL/kg lactated ringer for 5 days.

The bladders of the animals were evacuated manually twice daily until spontaneous voiding was established. Locomotor strength of the animals was rated with the Basso, Beattie, Bresnahan (BBB) scale (no movement to full range-22 points) and they were recorded by a blinded neurosurgeon after SCI was inflicted at Day 0, 7, 14, 21, 28, and 35 (2).

Also, the spinal cords at the level of ninth vertebra were removed on Day 35 following fixation with perfusion. The spinal cord was evaluated for neuronal injury (mild to severe-3 points), white matter cavitation / cyst formation (3 points, absent, few, more than 5 in one field), blood vessel ingrowth (absent, few, more than 5-3 points), edema (-/+), demyelization (mild to severe-3 points), inflammation (-/+), and hemorrhage (-/+) by a blinded-pathologist. Immunohistochemistry was used to detect expressions of Vascular Endothelial Growth Factor (VEGF) and Neuronal Growth Factor (NGF) protein in rat spinal cords. The levels of these factors' expression were assessed semiquantitatively by blinded histologists using a 3-point scale under the light microscope. In this scale, a score of 0 indicated no staining at all; 1 indicated scattered staining in neuronal tissue sampling; and 2 indicated intense staining.

Allen's spinal cord trauma method

The apparatus was a 10cm guide tube positioned perpendicular to the center of the spinal cord with an inner stainless steel rod (weighing 5 g). The animals were subjected to an impact of 50 g/cm² to the dorsal surface of the spinal cord.

Omegaven formula

The Omegaven Fish-oil emulsion used for the study was a 10% formulation. One hundred milliliters of this product contains the following components: eicosapentaenoic acid (EPA) (1.25-2.82 g), docosahexaenoic acid (DHA) (1.44-3.09 g), myristic

acid (0.1-0.6 g), palmitic acid (0.25-1.0), palmitoleic acid (0.3-0.9 g), stearic acid (0.05-0.2 g), oleic acid (0.6-1.3 g), linoleic acid (0.1-0.7 g), linolenic acid (<2g), octadecatetraenoic acid (0.05-0.4 g), eicosanoic acid (0.05-0.3 g), arachidonic acid (AA) (0.1-0.4 g), docosanoic acid (<0.15 g), docosapentanoic acid (0.15-0.45 g), D- α -tocopherol (0.015-0.0296 g), glycerol (2.5 g), and purified egg phosphatide (1.2 g). The osmolality is 308-376 mOsm/kg and the pH is 7.5-8.7. The daily recommended dose is 1 mL/kg (This whole data was taken from the original brochure of this product) (8).

Histology and Immunohistochemistry

The spinal cord tissues were fixed by perfusion using 4% paraformaldehyde (PFA) for histopathological and immunohistochemical analyses. Following perfusion, the spinal cords were removed. Each tissue sample was fixed in phosphate-buffered saline (PBS) and formalin. The tissue block was embedded in paraffin and 5 μ m-thick sections were cut. Sections were transferred to slides and coated with 3-aminopropyl-triethoxysilane (TESPA, SigmaA-3648). Each section was deparaffinized at 37°C overnight, then immersed in xylene for 5 minutes followed by ethanol for 5 minutes. This was repeated 3 times. Each slide was then rinsed with distilled water, submerged in 0.1% H₂O₂ for 30 minutes, and rinsed with distilled water again. For antigen retrieval, 0.1% trypsin was diluted in PBS to create a 0.1 μ g/mL solution and slides were immersed for 3 short periods (5 minutes each). Next, protein blockage was achieved by immersing the slides for 30 minutes in a mixture of 1% bovine serum albumin (BSA) in PBS. Each slide was washed in PBS for 3 periods of 5 minutes each, and then incubated overnight in 5 g/mL VEGF antibody specific for the 121, 165, 189 and 206 isoforms of VEGF (Oncogene VEGF /Ab-3; Cat. no. GF25, Calbiochem, USA). This step was followed by 3 more 5-minute washes in PBS. Slides were then placed in link solution (TM-125-BN (Lab Vision Corporation, UK) and incubated for 15 minutes at room temperature. Each was then washed 4 times in PBS. Next, each slide was immersed in labeling solution (streptavidin peroxidase; TS-125-HR, Lab Vision Corporation, UK) and incubated for 30 minutes, then rinsed 3 more times in PBS. Then 3,3' diaminobenzidine was applied as a chromogenic substrate and the slides were incubated until the desired reaction was achieved (approximately 5 to 7 minutes). After this, each slide was rinsed in tap water and stained with hematoxylin-eosin for 1 minute. Each immunohistochemically processed section was then covered with xylene. Sections 4 μ m-thick were placed on 3-aminopropylethylene-covered slides and deparaffinized at 60 °C for 1 hour. The slides were dewaxed in xylene and dehydrated in 96% alcohol. Then, sections were immersed in 10mmol/L citrate buffer (pH 6.0) and microwaved for 20 minutes for antigen retrieval. Slides were cooled to room temperature and rinsed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by further pretreatment with 3% H₂O₂ /distilled water for 20 minutes at room temperature. After a thorough washing in PBS, blocking solution (Lab Vision Co., Fremont, CA, 94539) was applied to block nonspecific antibody binding.

The sections were incubated with a primary antibodies mouse monoclonal VEGF(C-1) (Santa Cruz Biotechnology, Santa Cruz, CA, sc-7269, 1:100 dilution), rabbit polyclonal NGF(M-20) (Santa Cruz Biotechnology, Santa Cruz, CA, sc-549, 1:100 dilution) for 1hour at RT. After a 10-minute rinse in PBS, biotinylated goat anti-polyvalent immunoglobulin (Lab Vision Co., Fremont, CA, 94539) for 20 minutes, and the Streptavidin-biotin peroxidase complex for 20 minutes were applied at room temperature. 3, 3'Diaminobenzidine (DAB) chromogen was used for visualization of antigen-antibody binding. The sections were counter stained with hematoxylin, dehydrated, cleared in xylene and mounted on Entellan coverslips.

Statistical Analysis

All data were evaluated in a blinded fashion and expressed as mean \pm SD. Group data were statistically compared using Student's t test, Mann-Whitney U-tests and Chi-square test. Probability values <0.05 were considered to indicate a significant difference.

RESULTS

All rats completed the experiment successfully. Neither morbidity and mortality, nor any complication was observed. BBB scores were 0 in early period after SCI was inflicted. BBB scores progressively increased after Day 7 in both groups. BBB scores were significantly higher in group O when compared with control group after Day 7 in all times. (Figure 1).

Immunohistochemical analysis revealed that neuronal injury (in group C 2.66 ± 0.50 and group O 1.66 ± 0.50 ; $p < .002$) and edema was much more in control group (2.55 ± 0.52) when compared with group O (1.66 ± 0.50) ($p < .005$). Although, scores for white matter cavitation, demyelination and vessel ingrowth were lower in group O, there was no statistical significance (Figure 2A,B,C) (Table I). NGF expression was similar in two groups but VEGF expression in the control group was higher than in group O at both vessel ($p = .016$) and neuronal sites ($p = .019$). (Table I).

DISCUSSION

Lipid emulsions rich in fatty acids are already used for nutritional support in various patients, including surgical and critically ill patients (5,8,13). Many studies show that omega-3 PUFAs have significant therapeutic potential in SCI and our data suggest a similar result (3,9,11,15). Most preparations used at present contain a significant amount of soybean oil and therefore have a considerable omega-6 component. King et al. (11) showed that acute omega-3 PUFA administration after trauma as a neuroprotective treatment would confer clear advantages and deserves consideration as a promising innovative approach in SCI management. However, according to their results, omega-6 PUFA can worsen the outcome after SCI. The combined regime consisting of an acute bolus of omega-3 PUFA within the first hour after trauma, plus maintenance chronic administration of these fatty acids during recovery deserves consideration as one of the most promising approaches available for SCI management (9).

Table I: Results of Immunohistopathological Analysis of Spinal Cord Sampling

	Group C	Group O	P value
Neuronal injury*	2.66±0.50	1.66±0.50	.002
Demyelination	2.44±0.72	2.11±0.60	.261
White mater cavitation	2.55±0.72	2.00±0.70	.103
Blood vessel ingrowth	2.88±0.33	2.66±0.50	.298
Edema*	2.55±0.52	1.66±0.50	.005
VEGF (at vessel)*	1.88±1.11	1.66±0.52	.016
VEGF (at neuron)*	1.16±0.33	1.11±0.11	.019
NGF	2.66±0.50	2.44±0.72	.160

*: It shows the statistically significant parameter. **VEGF:** Vascular Endothelial Growth Factor, **NGF:** Neuronal Growth Factor.

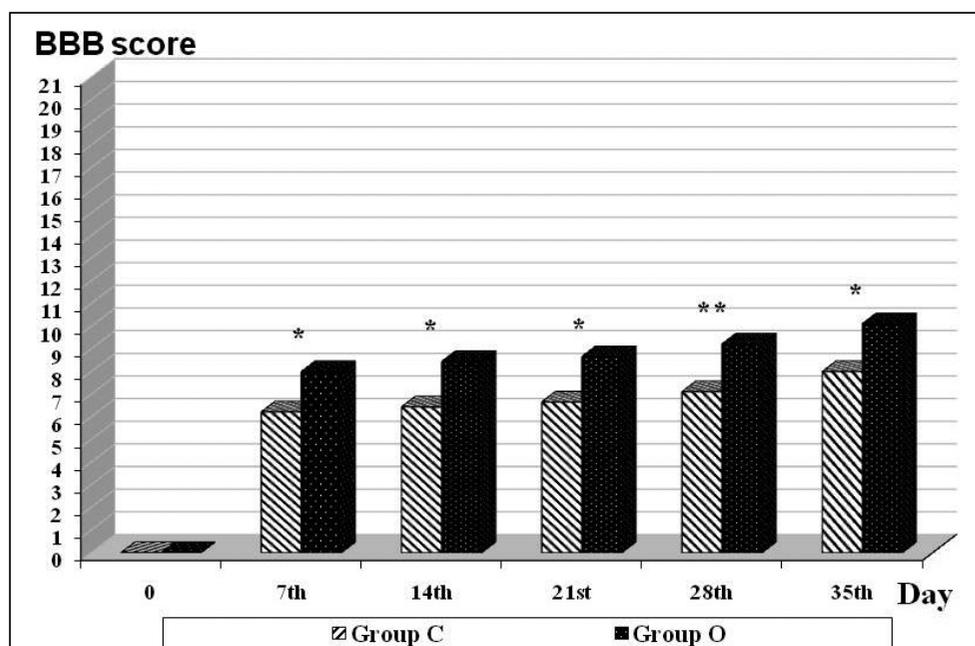


Figure 1: Evaluation of locomotor strength with BBB score.
*: p<0.05 and ** p<0.01 between two groups.

In this study we analyzed the effect of Omegaven, a product that consists of combination of two omega PUFA’s- omega-3 and omega-6. We found less neuronal injury and edema in the Omegaven group.

Neurotrophins are a group of small molecule polypeptides involved in both the development and maintenance of neurons. NGF can prevent the death of motoneurons after injury both in newborn and adult rats (16). Also, NGF receptor mRNA increased in adult motoneurons after axonal injury, indicating the protective role of NGF (12,14). Fumagalli et al. (7) suggest that the larger increase of NGF expression mediated by erythropoietin soon after the injury might explain, at least in part, the improved recovery of motor functions produced by erythropoietin compared to methylprednisolone and saline. Ikemoto et al. (10) reported that DHA enhances, whereas AA suppresses neurite outgrowth in PC12 cells. We could not find significant differences between saline and fish-

oil groups. This result may be dependant on the lipid content rates which include 0.1-0.4 g/100 mL AA. King et al. (11) also showed that omega-6 PUFA worsens outcome after SCI.

After injury to the nervous system, activation of VEGF and its receptors may restore the blood supply and promote neuronal survival and repair. Patel et al. (17) suggest a beneficial role of acutely administered VEGF in hastening neurobehavioral recovery after SCI. Recent studies have depicted the localization of VEGF and its receptors on neurons and astrocytes and they has been shown to induce neuritic growth and to provide neuroprotection particularly after ischemia or spinal cord injuries (4,19,20). Robson et al. (18) showed that omega-3 PUFA such as DHA, can be harnessed to promote neurite recovery after injury in vivo. Omega-3 PUFA has a marked neurite-promoting potential in neurons from adult and aged animals, seen both at the adult and aged stage (15). VEGF treatment resulted in earlier improvement

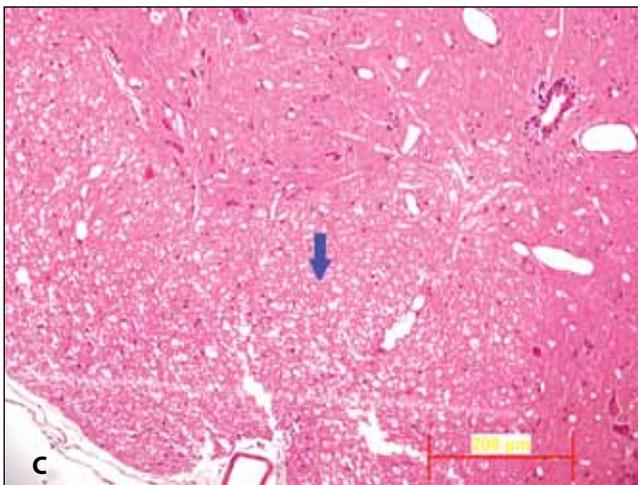
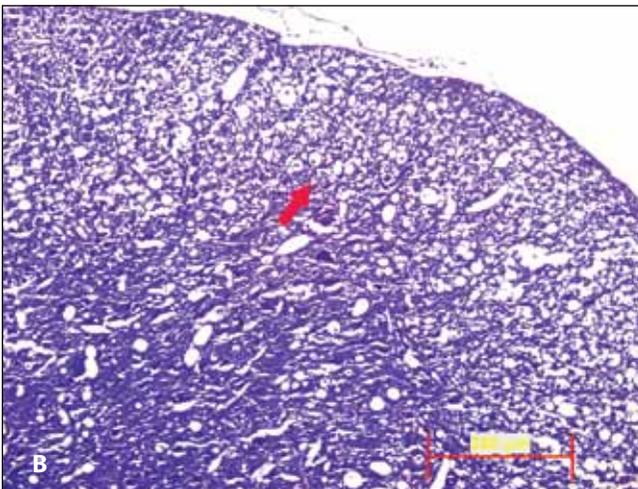
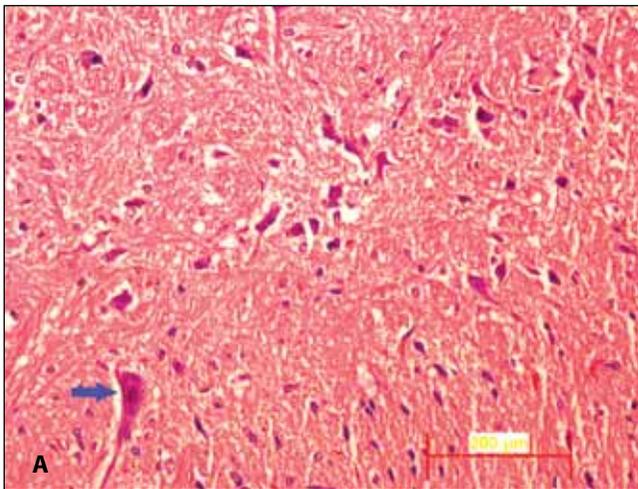


Figure 2: Immunohistopathological evaluations of spinal cords in Group O. **A)** Numerous intensely stained “red neurons” (arrow) are present in the spinal cord that shows neuronal injury. Haematoxylin & Eosin stain. **B)** Pale staining (arrow) of spinal cord shows decreased myelination. Vacuolizations are evident in the white matter. Luxol fast blue (LFB)-Cresyl violet stain. **C)** Arrow shows white matter cavitation / cyst formation in the white matter of spinal cord. Hematoxylin & Eosin stain.

in locomotor ability during the chronic phase of SCI (15). In our study, we found significant differences between the saline and fish-oil groups. In the saline group, vascularity was significantly more than the fish-oil group, and the better locomotor scores in group O can be explained with the less edema and less demyelination detected in this group.

At the cellular level, our results revealed that anti-inflammatory effect of Omegaven lead to a decrease in edema and a positive effect regarding the prevention of neuronal injury. Higher BBB scores were also achieved clinically. However, this treatment suppressed VEGF expression and did not influence angiogenesis.

In conclusion, we believe that nutritional support with fish-oil (Omegaven®) in patients with SCI will result in a better clinical outcome and increase the quality of the patient's life.

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