ORIGINAL ARTICLE

Effectiveness of Flaxseed Oil on Peripheral Nerve Regeneration Following Crush Injury of Sciatic Nerve in Rat: Behavioural Analysis and an Electron Microscopic Study

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ABSTRACT

Introduction: Peripheral nerve injuries (PNI) are a disabling injury as it often results in motor and sensory deficit with cognitive impairment. Flaxseed oil provides a good source of omega-3 fatty acid and it is believed to be able to protect the damaged nerve cell for successful nerve recovery. This study aimed to investigate a potential neuro-regeneration properties of flaxseed oil in treating the PNI. **Methods:** A total of 65 rats were separated into 4 groups: Group 1: Normal group (n=5), Group 2: Negative group (n=20), Group 3: Experimental group (n=20) and Group 4: Positive control (n=20), all the group were further divided into 4 groups (post-operative 7, 14, 21, 28 days, n=5 for each days). The functional restoration was assessed by walking track analysis (Sciatic Functional Index analysis-SFI) and toe spreading reflex (grading score). Electron microscope studies were performed on sciatic nerve to evaluate the regenerative process through morphologic and morphometric changes. **Results:** Oral administration of flaxseed oil (experimental group) at 1000 mg/kg body weight/day showed better recovery compared to negative control value. However, there was no significant difference in SFI and toe spreading reflex between positive (mecobalamin) and experimental group (flaxseed oil). Morphological and morphometrical findings indicated increases in the myelin thickness and myelin sheath layer after administration of flaxseed oil. **Conclusion:** The flaxseed oil supplementation could enhance the neurorestorative capacities of injured sciatic nerve.

Keywords: Sciatic nerve, Flaxseed oil, Behavioural analysis, Myelin sheath, Injury

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INTRODUCTION

Peripheral nerve injury (PNI) is described as trauma to the nerve as a result of an injection, gunshot wound, laceration and contusion (1). Although it is not a life threatening, nerve injury leads in partial or complete loss of motor, sensory and autonomous function with subsequent degree of disabilities. The incidence in developed countries is proximated to be between 13 to 23 per 100,000 people each year (2). Consequent to peripheral nerve injury, the local ion conduction blockage causing the target muscle unable to receive the message from the brain and reinnervation is retarded. Therefore, the functional motor deficit and the muscle become paralysed or weakened (3). In fact, prolonged denervation can lead to permanent muscle atrophy (4).

PNI was previously believed to be irreversible. However, axon of peripheral nerve is capable to regenerate almost readily depending on the severity of the injury (5). Currently, nerve grafting has become a gold standard to bridging the gap of injured nerve especially in severe injury (6). However, there are several issues regarding the nerve grafting method and these include length of the nerve gap to be restored, morbidity of donor and indication of appropriate nerve (7,8). Apart from that, the outcome is not always successful due to variation in the size and number of fascicle in the proximal and distal stump as well as expression of neurotrophic factor (9).

Flax plant (*Linum usitatissimum*) has the richest source of α-linolenic acid, the ω-3 polyunsaturated fatty acid (PUFA) which is an essential fatty acid for human (10). Flaxseed oil provides a good source of lignans a complex phenol and dietary fibres. Regarding the lignans content, flaxseed oil contains remarkable amount of secoisolariciresinol diglucoside as well as the present of matairesinol, isolariciresinol and pinoresinol (11,12). Chemical studies on flaxseed oil has revealed the bioactive molecule which can be used as a treatment

for lead kidney toxicity (13), cancer (14) and oxidative stress induced toxicity (15,16).

Therefore, the purpose of the study is to evaluate the sciatic nerve regeneration activity of flaxseed oil in Sprague-Dawley rat after nerve crush injury.

MATERIALS AND METHODS

Flaxseed Oil

Flaxseed oil (Kordel's, Catalent Australia) was purchased from a local pharmacy which containing 1000 mg of flaxseed oil.

Animal Handling and experimental design

All experimental procedures have been carried out in accordance to Management & Science University committee ethics with approval number of AE-MSU-034. 65 male Sprague Dawley rats with body weight ranging from 180-200g (2 months age), were supplied by the animal laboratory MSU. Animals were housed in rat standard polypropylene cages, lined with corncob bedding and kept under controlled temperature (20±2oC), humidity (40-60%) and 12 hours of light and 12 hours dark cycle. All rats were acclimatized for 7 days prior to the start of the study. A conventional rat diet (Specialty Feeds, Australia) and tap water supplied *ad libitum* (17). Animals were randomly divided into four groups of 20 rats each. Negative control group received daily oral administration of distilled water (10 mL/kg body weight per day), experimental group administered with flaxseed oil (1,000 mg/kg body weight per day) and positive control group administered with mecobalamin (130 µg/kg body weight per day) using esophageal feeding tube for 28 days post-operation period.

Sciatic Nerve Crush Injury

The rats were anesthetized with a mixture of ketamine and xylazine at doses 100 mL/kg body weight and 10 mL/kg body weight, respectively (17). The slitted skin exposed the left sciatic nerve through a gluteal muscle splitting. Using a fine watchmaker forcep no. 4, the sciatic nerve was compressed for 10s with constant pressure until white transparent band across the nerve was seen (18). The skin incision was sutured by Ethicon polyamide thread (Nylon, STERILER) and Mayo-Hegar's needle holder (Terumo, Terumo Corporation Japan). All rats had an accessed with conventional diet ad libitum and continuingly fed with distilled water, flaxseed oil or mecobalamin for 28 days post-surgery.

Walking Track Analysis

Walking track was set up with 8.2 x 42 cm dimension and white paper served as the base of the track (18). The rat's limb was dunked with Chinese ink and allowed to walk down the track, leaving the footprints on the white paper. The footprints were obtained before the surgery (day 0), and on day 7,14,21 and 28. The behavioural observation was based on the sciatic functional index (SFI) which derived from the measurement of footprints obtain from the walking track (19).

$$SFI = 38.3\ \frac{(EPL-NPL)}{NPL} + 109.5\ \frac{(ETS-NTS)}{NTS} + 13.3\ \frac{(EIT-NIT)}{NIT} - 8.8$$

The component of SFI includes print length factor (PL): distance from heel to toe, toe spread (TS): distance from the first to fifth toe and intermediate toe spread (IT): distance from the second and fourth toe were taken for normal (N) and experimental (E) foot.

Toe Spreading Reflex

The left hind limb function was evaluated and graded using 5-point scale of spreading reflex (Table I). The rats were held by their tail and lowered it down onto surface then carefully observed the toe spreading reflex (20). The observation based on comparison on the reflex activity between left toes (operated group) and right toes (unoperated group). A score of 0 was given when no visible abduction of the toes was observed, while a score of 4 indicated normal reflex which resemble the unoperated hind limb.

Table I: The spreading reflex by Gutmann 1942 (20)

TOE SPREADING REFLEX (Gutmann, 1942)	
GRADE	**CLINICAL SYMPTOMS**
Degree 0	Absence of any abduction (movement) in any digit
Degree I	Just visible spreading of the 4th toe alone
Degree II	Slight spreading of all three toes
Degree III	Spreading of all three toes less forceful than normal
Degree IV	Full spreading of all three toes (resemble unoperated right site)

Sciatic Nerve Isolation

The rats were anesthetized by overdosed mixture of ketamine (100mg/mL ketamine, Thailand) and xylazil (20mg/mL xylazine, Thailand) (17). Then, the rat was perfused through the heart (cardiac perfusion) with 300mL of normal saline followed by 300mL of 4% paraformaldehyde. Then, the muscles were separated by a gluteal-splitting muscle incision to disclose the sciatic nerve. The sciatic nerve was isolated by cutting 1cm distal from crushed site.

Transmission Electron Microscopic (TEM) Examination

The sciatic nerve was fixed in 4% glutaraldehyde of 0.1M phosphate buffer solution (PBS). The specimen were then washed with PBS three times, post-fixed for 2 hrs in 1% tetroxide osmium, dehydrated in graded concentration of alcohol and embed in Epon 812 resin (21). The semithin section were cut at 1 µm, stained with toluidine blue and viewed under the light microscope to observed the morphological changes of sciatic nerve. Ultrathin section were cut at 60-90 nm thick, stained with 1% uranyl acetate and 2% lead citrate and viewed under the Transmission Electron Microscope (TEM) (Leo Libra 120k, Zeiss) (22).

Statistical Analysis

All the data collected from the experiment was recorded

and analyzed using SPSS software for Windows (version 23.0, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with Tukey's post hoc analysis was used to compare differences among the groups. In each test, the data was expressed as the mean value ± standard deviation (SD) and differences were considered to be significant at *P<0.05*.

RESULTS

Toe Spreading Reflex

All rats demonstrated improvement in hind limb function during post-surgery period marked by abduction of any digits of the toe on the left injured hind limb (Table II). Rats treated with flaxseed oil and mecobalamin showed the earliest onset of recovery at day 3 and 4 respectively. As shown in Figure 1, the visible toe spreading reflex in negative control was not attained until 7 days after the injury. At 14 days after surgery, there was no significant different among the four groups. From 21 to 28 days after surgery, the toe spreading reflex value was significantly larger in experimental group than positive and negative groups. At days 13 and 15 post-operation, positive control and experimental group already achieved complete recovery in hind limb function respectively.

Table II: Mean toe spreading reflex score following sciatic nerve crush injury.

	Toe Spreading Reflex Value			
Group	Day 7	Day 14	Day 21	Day 28
Normal	4 ± 0	4 ± 0	4 0	4 ± 0
Negative Control	0.06 ± 0.08	0.45 ± 0.06	1.13 ± 0.18	1.86 ± 0.10
Experimental Group	0.56 ± 0.11	1.44 ± 0.18	2.20 ± 0.14	2.6 ± 0.12
Positive Control	0.91 ± 0.20	1.64 ± 0.23	2.45 ± 0.10	2.98 ± 0.08

Toe spreading reflex score of rats treated flaxseed oil or mecobalamin returned to pre-operation values 4-7 days earlier than negative control. Data presented as means ± standard deviation (n=5 for day 7, 14, 21 and 28 in all groups)

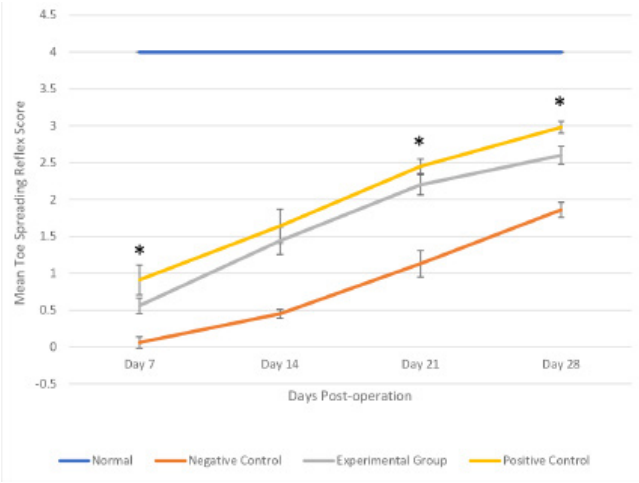

Figure 1: Graph of Mean Toe Spreading Reflex Score versus Days Post-operation. Toe Spreading Reflex performed before and after sciatic nerve crush injury. The curve plot showing the time-dependent change in the Toe Spreading Reflex score of rats in four groups. All data were represented as mean ± SD (n=5, * p< 0.05) vs. experimental group)

Walking Track Analysis

The restoration of hind limb function of sciatic nerve after the crush injury by the SCI analysis is shown in Table III. The print length was shorter at first and as recovery process occur the value increase to normal. All rats recovered consciousness after surgery. Walking tract analysis demonstrated that sciatic functional index value in each group increased with time (Figure 2). There was significantly different in sciatic functional index value at day 7 up to day 28 among those four groups ($p < 0.05$).

Morphologic and morphometric analysis

Electron microscope study of sciatic nerve section showed significant thickness of myelin sheath layer in the normal group and the axon consistently round in shape (Figure 3A). In the negative control group, the myelin sheath layer tremendously decreased in number followed by the disorientation in axon shape (Figure 3B and 3C). Increasing number of myelin sheath layer was observed in positive control group and experimental group. At days 28, the nerve of rats from positive control (Figure 3E) group already resemble the morphology characteristic of normal rats while negative control still under the recovery phase. Administration of flaxseed oil and mecobalamin significantly improved the morphology and morphometric parameter of sciatic nerve.

Table III : Sciatic function index (SFI) analysis following nerve crush injury

	SCI values				
Groups	Day 0	Day 7	Day 14	Day 21	Day 28
Negative Control	-22.14 ± 1.17	-65.87 ± 0.22	-58.36 ± 0.07	-49.89 ± 0.44	-35.15 ± 0.37
Experimental Group	-21.55 ± 1.10	-63.86 ± 0.17	-52.96 ± 0.17	-39.86 ± 0.40	-26.98 ± 0.30
Positive Control	-21.85 ± 0.67	-60.60 ± 0.17	-47.21 ± 0.28	-30. 15 ± 0.18	-24.14 ± 0.24

SCI analysis of negative control (untreated), experimental group (flaxseed oil) and positive control (mecobalamin) during pre-surgery and post-surgery. Results presented as a means ± standard deviation (n=5 for day 7, 14, 21 and 28 in all groups)

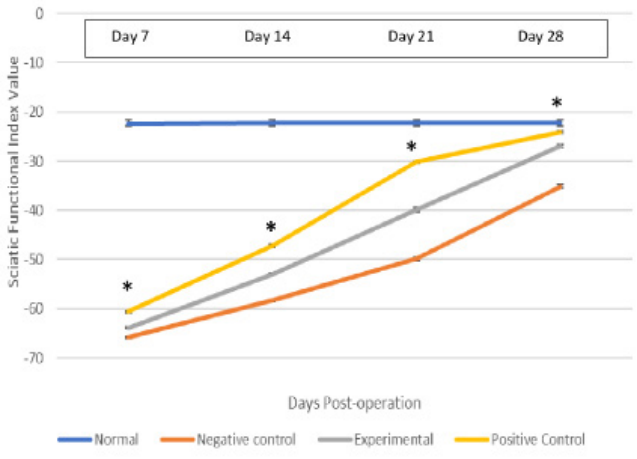

Figure 2: Graph of Sciatic Functional Index Value versus Days Post-operation. Sciatic Functional Index performed before and after sciatic nerve crush injury. The curve plot showing the time-dependent change in the Sciatic Functional Index score of rats in four groups. All data were represented as mean ± SD (n=5, * p< 0.05) vs. experimental group)

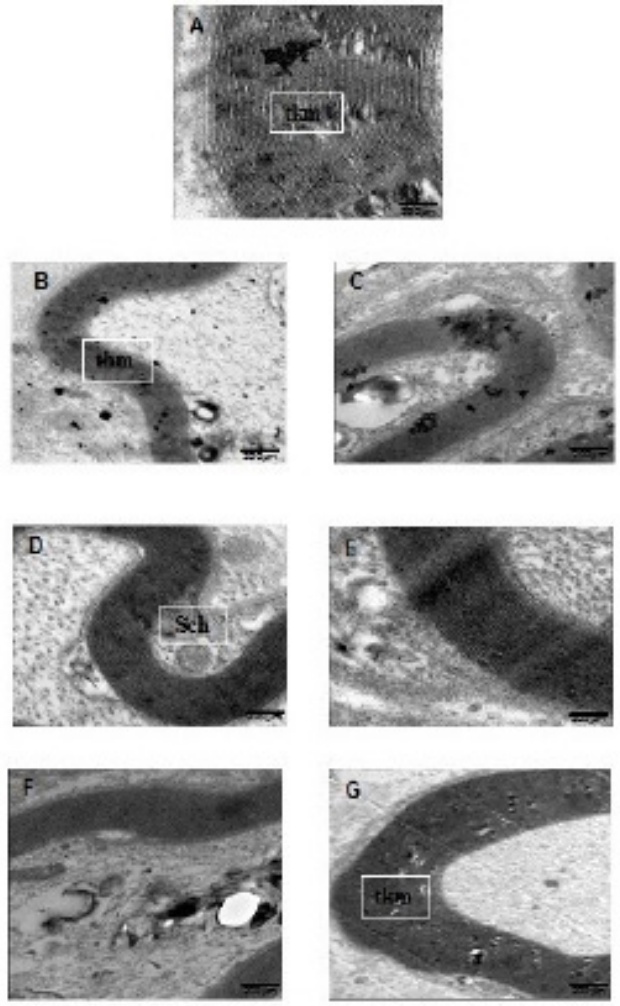

Figure 3: Photomicrograph demonstrate ultrathin section of sciatic nerve observed with Transmission Electron Microscope at various time points. A) Normal. B) Negative control: 14 days post-operation. C) Negative control: 28 days post-operation. D) Positive control: 14 days post-operation. E) Positive control:28 days post-operation. F) Experimental: 14 days post-operation. G) Experimental: 28 days post-operation. Scale bar = 50 µm. (tkm = thick myelin) (thm = thin myelin) (Sch = Schwann cell.

DISCUSSION

In the present study, rats model has been used to study the nerve regeneration and axonal sprouting after the partial or complete nerve injuries (23). Rodent possess neuroanatomical, functional and motor component that are almost similar to human. Therefore, rats are the most commonly used species for the modelling of neurological disease where they provide some advantages including the ability to perform advanced behavioural testing including walking track analysis and toe spreading reflex (24). The results indicate that the supplementation of flaxseed oil is enable to promote nerve regeneration by expedite the functional recovery and improve the morphology and morphometric outcomes.

Nerve crush injury consistently demonstrated and is a long-established axonotmetic model in experimental regeneration studies. In spite of the complete interruption of the nerve axon and surrounding myelin layer, the anatomical continuity is remained which provide a path for regeneration of axon and subsequent reinnervation of target organ (5). However, delays in nerve regeneration can lead to denervation of target organ and potential permanent atrophy. Therefore, a quick regeneration process is pivotal to achieved satisfactory functional outcomes (25).

Sciatic nerve responsible for hind limb function in rat can be assessed quantitatively by sciatic functional index analysis and score of toes spreading reflex. The footprint obtained by the gait analysis consistently demonstrated the functional outcome of the treatment received by the rats (26). While, the abduction of any digit of toe provide a reliable marker to assess the extent of injury and monitoring the recuperation (27). The clinical relevance of this method is marked by the regeneration of nerve, reestablishment of nervous circuit and restoration of motor function (28).

In the present study, modified Watchmaker's forceps were used to induce a sciatic nerve crush injury as it is adaptable, affordable and proven to be clinically relevant. Forceps with different separation distances (calibrated ignition gauge or spacer) inserted at closing point was used to standardized the pressure and it is viable to achieved mild, moderate, and severe injuries that can be categorized histologically (29). Flaxseed oil and mecobalamin provide a quicker onset and complete functional recovery in comparison with untreated rat (negative control). It was found that complete recovery of the nerve was attained at day-18 in the experimental group and day-15 in the positive control. Hence, flaxseed oil shortens the time taken for complete functional recovery compared to that of the negative control. However, this reduction is not statistically significant when compared with positive control. These findings were consistent in both toe spreading reflex score and walking track analysis.

The morphology and morphometric parameter are frequently used to evaluate the regeneration process of injured nerve. The following parameters provide a reliable indicator to study on nerve regeneration because they parallel with functional recovery outcome (30). The thickness of myelin layer was assessed in this study. The loss of myelin layers after the sciatic nerve injury might be due to damage of myelin sheath as a result of nerve cells apoptosis and degeneration of myelin sheath around the axon (21). However, the myelin thickness increases after the administration of flaxseed oil and mecobalamin indicated that the degeneration of nerve fibre is reversible. The presence of small nerve fibres reflects the undergoing regeneration process however, it was yet an unfinished process as the features of degenerative process were still present. According to Raducan et al., (29) the process of myelination is still incomplete and regeneration still occur one-month post

injury even though the myelin layer had increased over time.

The flaxseed oil is known to have the highest dietary sources of ω-3 poly-unsaturated fatty acid among plant sources (31). Flaxseed oil consist of three major component: 53% α-linolenic acid (ALA), an ω-3 PUFAs, a good source of dietary fiber and complex phenol namely lignins (32,33). It has been reported that, the degree of ALA conversion to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is 5-10% and 2-5% respectively (34) whereby the body unable to synthesis the ω-3 poly-unsaturated fatty acid and therefore must be obtained through diet. The accelerated of sciatic nerve regeneration may be due to the action of α-linolenic acid and its derivatives such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (35,36) where these components are responsible for the membrane structure in myelin sheath of central and peripheral nervous system (37).

CONCLUSION

From this study, we have shown that the flaxseed oil expedites the nerve capacities to regenerate after sciatic nerve injury. Moreover, there was significant difference in the functional hind limb recovery, morphologic and morphometric changes after the administration of flaxseed oil and mecobalamin. We conclude that, α-linolenic acid is one of the major profile that is implicated in nerve regeneration.

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