ORIGINAL ARTICLE

Effect of Tocotrienol Rich Fraction (TRF) On Muscles Reinnervation After Sciatic Nerve Crush Injury In Rats

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ABSTRACT

Introduction: Muscle denervation is a process where muscles lose nerve supply due to neural damage and this may lead to paralysis in human. Muscle denervation is mainly caused by peripheral nerve injuries especially in the lower extremities that resulted in devastating effect on human daily functions and routines. Tocotrienol Rich Fraction (TRF) consist of 75% of tocotrienols have shown potential neuroprotective properties. The objective of this study is to observe motor coordination and histological characteristics on muscles that underwent sciatic nerve crush injury and supplemented with TRF. **Methods:** A total of 104 Sprague-Dawley rats were divided into four groups; normal group (n=8) with no sciatic nerve crush injury, negative control (n=32) with sciatic nerve crush injury at hindlimb without treatment, positive control (n=32) sciatic nerve crush injury treated with 500 µg/kg/day of methylcobalamin, and experimental group (n=32) of rats that underwent sciatic nerve crush injury and treated with 200 mg/kg/day of TRF. **Result:** Skeletal muscles which located at hind limb; Soleus Muscle and Extenstor Digitorum Longus Muscle (EDL) muscle have shown an increasing in weight when it is supplemented with TRF 200 mg/kg/day and improved myelin layer of nerve. **Conclusion:** This study showed that TRF has the potency to improve reinnervation rate and neuron supply in hind muscle.

Keywords: Muscle denervation, Sciatic Nerve, Motor coordination, Tocotrienol rich fraction

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INTRODUCTION

Muscle denervation occurs when there is a loss of nerve supply to the muscle and it can be caused by several factors in and out of control that gives negative impact to life (1). Denervated muscles will undergo atrophy, a degeneration of cells. Muscle denervation can occur in many clinical settings, including trauma or physical injury, diabetic neuropathy, alcoholic neuropathy, pernicious anemia, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy, Charcot-Marie-Tooth disease, and viral infections such as polio (2).

Injuries include peripheral nerve injury (PNI) can also lead to muscle denervation that cause weakness or paralysis. The cases of PNI in developed countries are estimated between 13- 23 in a population of 100,000 per year (3). This may result in partial or total loss of motor, sensory and autonomic body functions. Axons have the capability to regenerate after nerve injury and it provides a proper pathway to reconnect with their targets (3). Any therapeutic medication that can speed up axonal regeneration will increase the functional recovery (3). However, inadequate way of treatments may cause neuroma and scarring of the injured nervous tissue, which will inhibit nerve regeneration (3). At present, commercial drugs may be used as treatment of PNI, but it may be associated with severe side effects such as liver and kidney disorders (4,5).

The use of herbs and natural products are known to have many beneficial effects in pharmacology research at present and in the future (5,6). One of these natural products is of the species *Elais guineensis* or palm oil. It is one of the natural sources where the extraction of palm oil or TRF that contain 75% of tocotrienols which has been reported to be neuroprotective effect (8). Malaysia is one of the largest producers and exporters of oil palm in the world (9,10).

Thus, treatment using TRF from palm oil extraction can be an alternative medicine to promote nerve regeneration as well as innervation of motor end plates due to high

content of Vitamin E and essential fatty acids.

Previous study has shown a pharmacological potential of TRF in neurodegenerative disorder. However, there is still little evidence of TRF effects on motor function or reinnervation of muscle after peripheral nerve injury. TRF can be one of natural supplement as previous studies have shown TRF have an effect in protecting body from oxidative liver damage (11).

Hence, this study on TRF in palm oil is to investigate muscle reinnervation in EDL muscle, soleus muscle after sciatic nerve injury as there is a chance of TRF to cross brain barrier due to lipid soluble content of vitamin E, besides its essential fatty acid content such as palmitic acid and linoleic acid.

MATERIALS AND METHODS

Animal Experiment

All experimental procedures have been performed in accordance with animal ethics that were approved by Management and Science University (MSU) Ethic Committee (Ethical approval number: AE-MSU-034). Hundred and four healthy Sprague Dawley rats age 8 weeks old, weighing 200 ± 50g were housed in MSU animal house with control temperature (22 C- 24 °C) under 12 hours light and 12 hours dark cycles and also had free access to food and water. Rats were categorized randomly into four groups:

Group 1. Normal group (n=8). Mechanism of sciatic nerve crush injury was not given to this group and it served as a control for behavioural and histological study. The rats were fed ad libitum.

Group 2. Negative control (n=32) were further sub-divided into four groups (7, 14, 21 and 28 days) and each group consisted of 8 rats, respectively. Mechanism of sciatic nerve crush injury were given and fed with only conventional ad libitum and no treatment were needed.

Group 3. Positive control (n=32) were subdivided into eight for four groups of 7, 14, 21 and 28 days. Mechanism of sciatic nerve crush injury were given and the rats were treated with methylcobalamin (nerve injury treatment drug) (500 µg/kg/day) by oral gavage using stainless steel feeding needle.

Group 4. Experimental group (n=32). The rats also were divided into four groups of 7,14, 21 and 28 days. Mechanism of sicatic nerve cruch injury were given and also supplemented with Tocotrienol Rich Fraction (TRF) with dosage of 200 mg/kg/day by oral gavage.

Introdcution to Sciatic Nerve Injury

Rats were anesthetized with a combination dose of ketamine (80 mg/kg) and xylazine (10 mg/kg). Right hind limb of rats served as the control and were anesthetized intramuscularly. Sciatic nerve injury was induced at the right hind limb. Left hind limb area was clean-shaven and sterilize with betadine. A 2 cm incision was made over lateral aspect of the hind limb and muscles were separated without cutting the muscle fiber in order to expose the sciatic nerve. The sciatic nerve was crushed about 1 cm proximal to the division of sciatic nerve. A consistent pressure for 10 seconds was applied by using sterile Watchmaker's forceps. The complete crush of sciatic nerve can be observed when a uniform transparent zone appears after several minutes. The skin and muscle incision was sutured by using 9/0 and 10/0 nylone and were monitored until they recovered from the anaesthesia. After recovery, each rat was returned into their cages according to their groups and they were examined daily.

Muscle Weight Measurement

At the end of the sciatic nerve injury and drug administration experiment, rats were euthanized at different time points: 7, 14, 21 and 28 days. The rats were sacrificed by cervical dislocation. Soleus muscle and EDL muscles were harvested the weight was measured. Wet muscle weight was measured after sacrifice to indicate the degree of denervation as the denervated and contralateral control side were compared. The muscles were photographed to see gross picture of sample after treated with TRF.

Behavioural Study

Rotarod Testing

After surgery, motor coordination was evaluated by using rotarod apparatus in which all groups of rats were placed on rotating drum with the speed increased from 5 to 25 revolutions per minute (rpm). The rats were habituated at 5 rpm for 1 minute before data evaluation. The latency to fall from rotarod was measured in seconds (s) and each rat was given three trials on each day after treatment. The apparatus was disinfected with 70% ethanol solution in between the sessions and wiped with tissue paper. The motor performance was evaluated on days 7, 14, 21 and 28 after surgery.

Histology Study

Muscle Histology

Rats were euthanized at different time points: 7, 14, 21 and 28 days post injury. Soleus muscle and EDL muscle were harvested for histological processing and analysis. All tissues harvested were fixed in 10% formalin for 24 hours before tissue processing was carried out. The fixed tissue was embedded in paraffin wax and sectioned into 5 µm sections using using a rotary microtome.

The muscle sections were stained with Hematoxylin & Eosin staining to assess motor endplate of muscle reinnervation and neural activity in the hippocampus at different time points. The stained slides were observed under light microscope after mounted with DPX.

Electron Microscopic Study

Transmission Electron Microscope

The sciatic nerve was also harvested at the different

time points of which the rats were sacrificed in order to evaluate the regeneration of sciatic nerve. The sciatic nerves were fixed in mixture of 3% glutaraldehyde in Phosphate Buffered Saline (PBS) for 24 hours at 4 C and it was post-fixed in PBS with 1% of osmium tetroxide for 1 hour and dehydrated in a series of gradient alcohol. The tissue was dipped in propylene oxide and embedding material mixture. The tissues were sectioned transversely at 70 µm thick by using an ultramicrotome. Then, the sectioned tissues were stained with 1% of uranyl acetate and 3% of lead citrate for 5 minutes and the sections were observed using a transmission electron microscope.

Statistical Analysis

The muscles were weighed, behavioural and histological analysis was carried out to compare the effect of TRF and methylcobalamin. Mean of muscles weight and myelin layer number were calculated by using one- way ANOVA while histology of muscles and nerve guided by microscopic observation. Data collected was analysed by using SPSS software version 25.0.

RESULTS

TRF Increased Muscles Weight Compared to Negative Control Group

Rats that were given TRF by oral administration have shown an increased amount of soleus muscle weight compared to negative control group which are shown in Figure 1. All the groups that have been sacrificed with different time points have shown the changes in soleus muscle weight where rats treated with TRF have shown an improvement of muscle weight from day 1 to day 28. The mean of muscle weight (Figure 1) have increased in experimental group compared to rats that have not been given any treatment which it is based on mean standard error of mean (SEM) ($p < 0.05$).

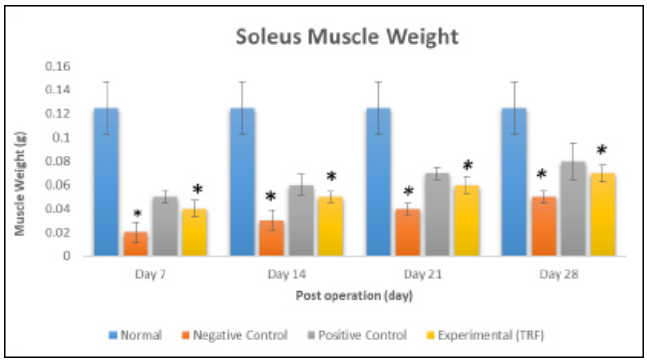

Figure 1: : Soleus muscle weight (g) for normal, negative control, positive control, and experimental groups at different time intervals post-injury days 7,14, 21 and 28. ($p < 0.05$).

EDL muscle weight also have been observed to have increased in weight after treated with TRF (Figure 2). Mean of EDL muscle weight was calculated (Figure 2) based SEM and it have shown an increased in amount in positive control group and experimental group compared to negative control group. Although the muscle weight in positive control group was increased compared to experimental group, the recovery rate of TRF and methylcobalamin were quite similar as there is no significant difference between both groups. Muscle reinnervation rate is faster in experimental group when compared to negative control group as the weight was increased in experimental group (Figure 2). There is no significant difference between experimental group and positive control group (Figure 2). Experimental group and negative control group have significance difference ($p < 0.05$).

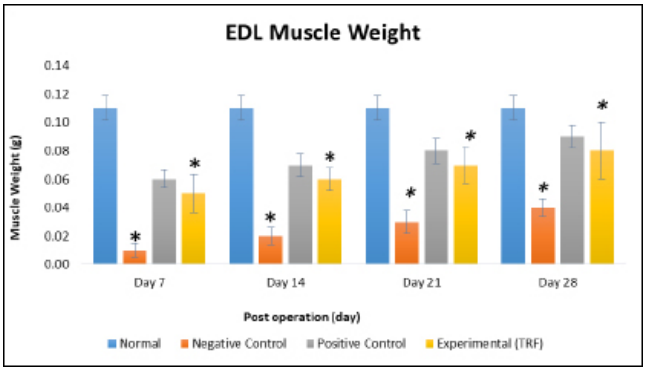

Figure 2: EDL muscle weight (g) for normal, negative control, positive control, and experimental groups at different time intervals post-injury days 7,14, 21 and 28. ($p < 0.05$)

Behavioural Study

TRF Increased Motor Coordination

Motor coordination of rats were assessed by rotarod testing. In Figure 3, it is shown that rats treated with TRF have an increased motor coordination in rotarod test compared to rats that have not given any treatment. The motor coordination also has improved from day 7 to day 28 when it is treated with TRF. Negative control group also have improved its motor coordination rate where it is increased by day 28 but the recovery process was slower compared to experimental group and positive control group. In positive control group, the rate of motor coordination also slightly increased but have no significant difference with experimental group.

Histologic Study

TRF Improved Muscle Fibers

Based on Figure 4 and Figure 5, soleus muscle and

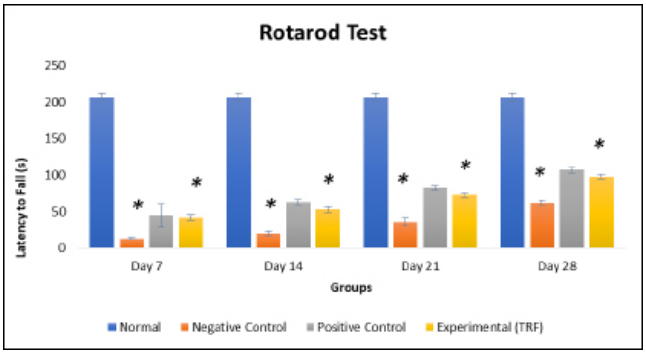

Figure 3: Rotarod test for normal group, negative control, positive control, and experimental group at different time intervals post-injury days 7, 14, 21 and 28. ($p < 0.05$)

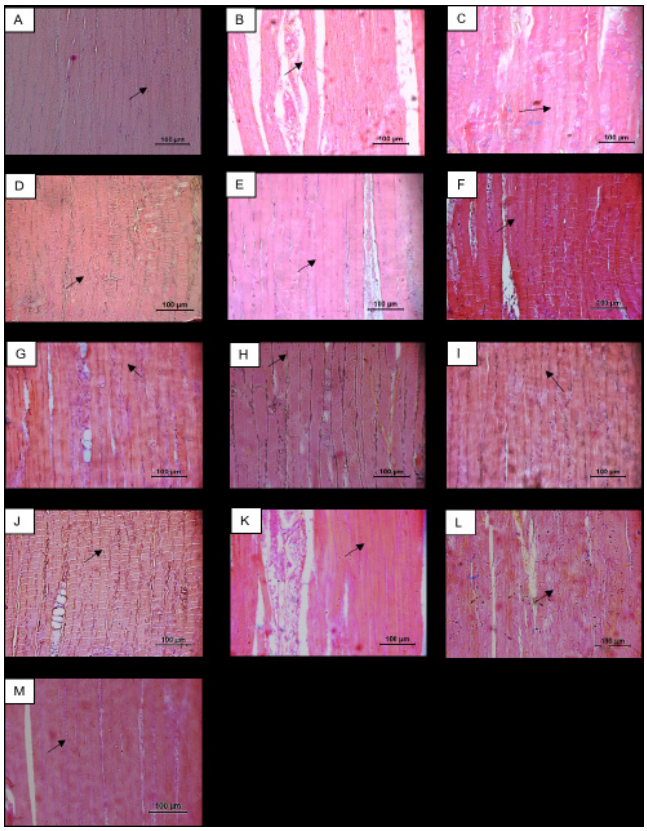

Figure 4: Photomicrographs showing longitudinal section of soleus muscle stained with H&E examined under 20x magnification. A) normal. B) negative control 7 days post-injury. C) negative control 14 days post-injury. D) negative control 21 days post-injury. E) negative control 28 days post-injury. F) positive control 7 days post-injury. G) positive control 14 days post-injury. H) positive control 21 days post-injury. I) positive control 28 days post-injury. J) experimental group 7 days post-injury. K) experimental group 14 days post-injury. L) experimental group 21 days post-injury. M) experimental group 28 days post-injury. (↘) shows striations of muscle fibers.

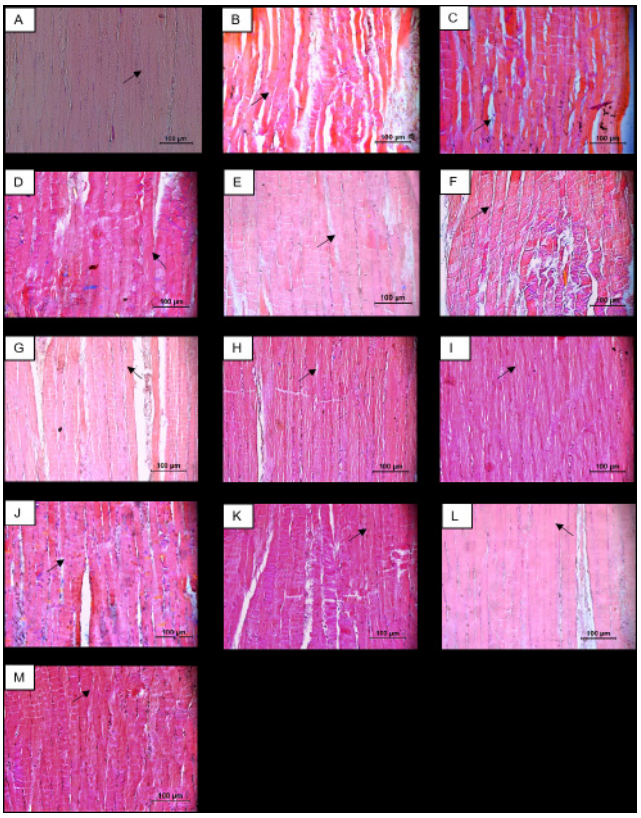

Figure 5: Photomicrographs showing longitudinal section of EDL stained with H&E examined under 20x magnification. A) normal. B) negative control 7 days post-injury. C) negative control 14 days post-injury. D) negative control 21 days post-injury. E) negative control 28 days post-injury. F) positive control 7 days post-injury. G) positive control 14 days post-injury. H) positive control 21 days post-injury. I) positive control 28 days post-injury. J) experimental group 7 days post-injury. K) experimental group 14 days post-injury. L) experimental group 21 days post-injury. M) experimental group 28 days post-injury. (↘) shows striations of muscle fibers.

EDL muscle supplemented with TRF have shown an improvement whereby muscle fiber have arranged accordingly compared to negative control group which have thick perimysium layer, muscle fibers are not well arranged and loss of muscle striations. Experimental group have shown faster recovery rate where it widened layer of perimysium, arranged muscle fiber and reduced loss of muscle striations.

For 7 days post-injury, there is degeneration of muscle fiber shown in negative control group whereby the arrangement of muscle fiber was scattered. Besides that, wide layer of perimysium can be seen between bundles of muscles. Muscles striation also have loss in negative control group. Different in experimental group, it shown an arranged muscle fiber with slightly wide layer of perimysium with normal muscle fiber in between. The loss of muscle striations also were quite minimal compared to negative control. Histological findings of muscles in positive control group are quite similar with experimental group in its arrangement and muscle striations. Same histological findings can be found on day 14 after injury with scattered arrangement of muscle fibers. There is also wide layer of perimysium at degenerated area and loss of muscle striations also can be seen in negative control group. For experimental group and positive control group, perimysium layer was same in day 7 and its arrangement have improved. The muscle also become striated.

In day 21 and day 28, same histological pattern can be seen in muscles whereby the arrangement of muscle fiber has been improved where it is arranged accordingly almost similar to normal muscle arrangement.

This shown that the rate of recovery process has increased in day 28. Perimysium layer also become thinner compared to groups in day 7 and 14 where there is normal muscle in between muscle fiber. Loss of muscle striations have also have been reduced compared to negative control group (Figure 4 and Figure 5).

Electron Microscopic Study

TRF Increased Myelin Layers at Sciatic Nerve

Based on Figure 6 and 7 cross section of sciatic nerve was observed to identify the number of myelin layer after supplemeted with TRF through TEM. The cross section was observed at the injured site for 14 and 28 days as

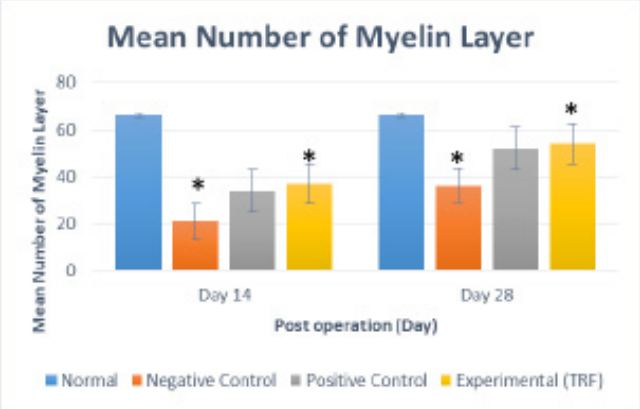

Figure 6: Number of myelin layer for normal, control negative, control positive and experimental groups based on SEM for mean number of myelin layer at different time intervals post-injury days 14 and 28. ($p < 0.05$)

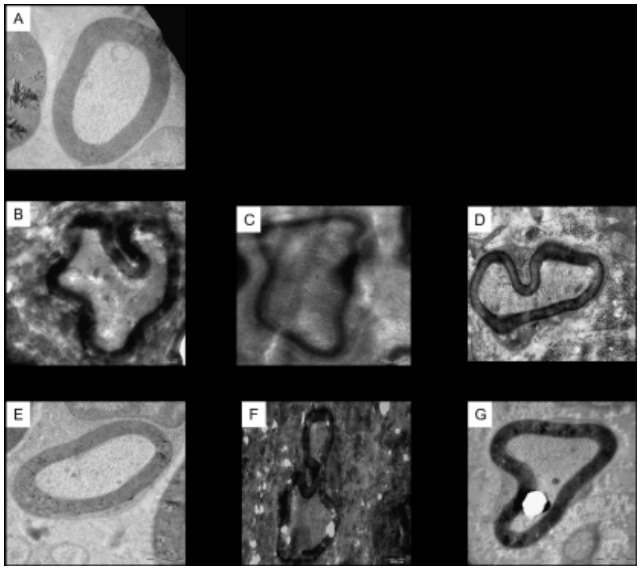

Figure 7: Photomicrographs showing cross section of injured sciatic nerve through TEM. A) normal group. B) negative control 14 days post-injury. C) negative control 28 days post-injury. D) positive control 14 days post-injury. E) postive control 28 days post-injury. F) experimental group 14 days post-injury. G) experimental group 28 days post-injury.

there is a diference number of myelin layer.There is no difference reduction in myelin layer number between 7 and 14 day same goes between 21 and 28 days. Hence, the result was observed in 14 and 28 days to compare recovery process of myelin layer.

Based on the results, experimental group has faster recovery process of myelin layer as the mean number of myelin layer increased over time (Figure 6). For 14 days post-injury, there is reduction of myelin layer whereby the mean number of myelin layer decreased compared to normal group. It was significantly difference from the mean number of myelin layer at 28 days post-injury. Myelin layer shown faster recovery in experimental group compared to positive control group as there is significant difference in mean number of myelin layer. Figure 7 represented TEM of sciatic nerve showing histological features of myelinated nerve fibers. Experimental group have improved its myelination compared to positive control group. Thicker myelin sheath was observed in normal group ($p < 0.05$).

DISCUSSION

In the present study, oral supplementation of TRF at a dose 200 mg/kg/day have potential in improve nerve regeneration after sciatic nerve crush injury. Previous studies have shown that administration of TRF for 200 mg/kg/day gives neuroprotective effect due to its antioxidant properties (12). There are many techniques used by scientist in developing sciatic nerve injury model. One of the method is by using Watchmaker's forceps. It was used in this study as it is clinically relevant to induce sciatic nerve crush injury (4,13). Sciatic nerve was crushed by applying 10 seconds pressure at second notch of the forceps causing crushing force at nerve segment. Based on previous studies, by applying pressure on nerve for 5 to 15 seconds at third notch can caused compression lesions to the nerve which can be differentiated histologically (4,13,14).

Based on results of behavioural and histologocial study, muscle reinnervation process and motor coordination rat can be seen in rats supplemented with oral administration of TRF 200 mg/kg/day compared to negative control group and positive control group. This have been demonstrated in result of muscle weight, behavioural analysis and histological analysis which have shown rapid muscle reinnervation and peripheral nerve regeneration rate with significant recovery process. Muscles weight was observed to see the atrophy and hypertrophy of muscle fibers. Atrophy of muscle fiber occur in both soleus and EDL muscle in negative control group. The loss of muscle weight does not reflected to loss of body weight as the rats was retained with proper nutrition. The maintained body weight of rats indicated that rats have been supplied with good nutrition during treatment (15).

Based on result, muscle fibers of soleus and EDL muscle underwent progressive atrophy after sciatic nerve crush injury. Prolonged denervation of muscle fibers occurred as it does not receive any treatment for 28 days and have increased connective tissue as well as small atrophic muscle fibers (15). Prolonged denervation caused neuromuscular alterations which resulted to reduction of muscle mass and size of muscle fiber.

Motor coordination and balance were assessed by using rotarod test and it is usually used in testing of neurological deficits in experimental rodents (16). The time taken for rats to fall from rotatord were different in each group. It is shown that the motor coordination rats were increased after treatment as the locomotor activity was also increased. In response to sciatic nerve crush injury, the behaviour was completely distorted as the rats was paralysed and cannot use up the muscles at left

hindlimb.

Previous studies have shown that the number of motor neurons was decreased as rats undergo process of Wallerian Degeneration where macrophages started to clear all debris at injured site (15). In our study, it have shown that the motor performance was improved after supplemented with TRF. During Wallerian degeneration, muscles were paralysed as nerve does not regenerate (15). This can be seen in microscopy result in negative control group where muscles denervated. The perimysium become wider and arrangement of muscles are not arranged accordingly. This may be due to deposition of fats and neurovascular bundles that were formed in perimysium layer (15). Nerve injury reduced neuromuscular transmission from nerve to the muscle as the impulses were unable to reached neuromuscular junction due to loss of contact with injured axons. Muscles that underwent atrophy can be seen microscopically where the muscle structures damaged and it is important in clinical problem (15).

The reinnervation process have been shown in morphological changes whereby the perimysium become thinner and arranged accordingly. The muscle atrophy has been reduced where most of muscle fibers have received neuronal transmission from regenerated nerve. Each muscle fibers have motor endplate that supply the nerve. When muscle denervates, the motor endplate have reduced causing muscle to loss nerve impulse and cannot contract (16). When it is treated, the reinnervation process occur whereby motor endplate may have increased causing muscle to move as it received neuromuscular transmission through motor endplate.

Sciatic nerve histology has been seen microscopically by TEM to observe myelination of nerve fibers. An increased in number and thickness of myelin layer signifies a nerve regeneration as myelin sheath is important in protecting nerves and act as electrical insulator (14). Decreased of myelin layer number due to nerve degeneration can cause nerve to lose its function. Myelin layer number have increased after supplemented with TRF shown that there is myelination occur based on mean myelin layer number and histological features of thickness of myelin layer.

In response to treatment of muscle reinnervation, Vitamin E is important in maintaining neurological structure and function. TRF are naturally occurred vitamin E which can be consumed by humans with no adverse effects documented (17,18). TRF have significantly increased muscle reinnervation after peripheral nerve injury. It improves muscles reinnervation, nerve regeneration and moto coordination for faster recovery of crushed sciatic nerve.

CONCLUSION

In conclusion, oral administration of 200 mg/kg/day of TRF aid in enhancing muscle reinnervation after sciatic nerve crush injury. This may be contributed by the presence of major (75%) active compound tocotrienol of TRF which is needed for neuron supply to the muscle at motor endplate. It also contained antioxidant that may have protective effect in protecting nerve from undergo neuronal damage.

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