CHAPTER IV
IN VIVO EFFECT OF NATURAL PRODUCTS LOADED ZEIN BASED POLYMER ON NEUROPATHY IN DIABETIC CONDITION

1. Introduction

Peripheral neuropathy, one of the most common complications of diabetes mellitus, occurs in more than 50% of diabetic patients (Charnogursky et al., 2014). It has been reported that most neuropathy affects all types of nerve fiber including large fiber sensory, small-fiber sensory, autonomic, and motor nerves. In addition, neuropathy may produce either symmetrical or asymmetrical changes on both sides of the body. The most commonly found is the sensorimotor peripheral neuropathy. Neuropathy is characterized by a progressive loss of nerve fiber, axonal degeneration and segmental demyelination with a slowing of nerve conduction velocity. The sensory and motor deficits are also observed (Shaikh and Somani, 2010). The clinical manifestations of diabetic neuropathy are varied depending on the affected nerve. To date, there is no definitive course of treatment available for diabetic neuropathy. The only established intervention to treat diabetic neuropathy is to normalize glucose metabolism. Therefore, the effective therapeutic strategy against neuropathy is required.

A substantial of evidence points out that diabetes induces peripheral neuropathy via several mechanisms including the increased oxidative stress and aldose reductase, reduced sodium-potassium ATPase levels, altered fatty acid metabolism, accumulation of advanced glycated end products, accelerated neuronal apoptosis, immunological alterations, and changes in blood flow (Perkins and Bril, 2003). Interestingly, the substances possessing antioxidant activity and aldose reductase suppression effect such as quercetin (Goodarzi et al., 2006) and tomato extract (Ali and Agha, 2009) could mitigate neuropathy in STZ-induced diabetic rats (Kandhare et al., 2012; Kuhad and Chopra, 2008). Since transdermal drug administration provides more advantages than oral administration including the avoidance of first pass effect, ease of application, less drugs fluctuation, achievement of efficacy with total daily lower dose and exert more site

specific, the administration of quercetin and tomato extract to mitigate neuropathy in diabetic condition via transdermal route has gained attention. Recently, electrospun polymeric nanofiber has been shown to improve the transdermal delivery system effectiveness (Son et al., 2014). Therefore, it was hypothesized that the electrospun polymeric nanofiber loaded with natural products such as quercetin or tomato extract could successfully enhance the recovery of neuropathy in diabetic condition. Since no data are available until now, the effects of electrospun polymeric nanofiber loaded with quercetin or loaded with tomato extract on the recovery of peripheral neuropathy in streptozotocin-induced diabetic rats were investigated.

2. Materials and Method

2.1 Chemicals

Streptozotocins, Zein, Quercetin, DMF (N, N-Dimethylformamide, C3H7NO) and other chemicals reagents and solvents were purchased from Sigma–Aldrich Co., USA. One touch glucometer (Accu-chek sensor) of Roche Diagnostics, Germany was obtained from Bayer Diagnostics Thailand Ltd. All substances used in this study were in Analytical grade.

2.2 Induction of Diabetic Neuropathy

Adult male Wistar rats, weighing 180-200 g, were used for this study. They were maintained according to the Guidelines of Animal Care described by Animal Center, Faculty of Medicine, Khon Kaen University. This study was also conducted under the review and approval of the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand. The record number is AEKKU 11/2552 and the reference number is 0514.1.12.2/8.

All animals were fasted overnight and induced diabetes mellitus by a single shot injection of streptozotocin (STZ) at dose of 50 mg kg-1 in 0.1 M citrate buffer, pH 4.5 via intraperitoneal route. STZ injection rapidly produced the characteristic signs of diabetes, such as increased intake of water and food, frequent urination and increased blood glucose concentration. Forty-eight hours later, blood samples were collected and glucose levels were determined to confirm the development of diabetes. Only the

animals which showed blood glucose levels >250 $mg.dl^{-1}$ were used in the experiment.

Since DPN occurs as a result of both neural and vascular derangements, the experimental neuropathy in this study was performed by inducing both neural and vascular disturbances by crushing right sciatic nerve with hemostatic forceps for 30 seconds at the upper one third of sciatic nerve. In brief, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). An incision was made at the back of the thigh and the right sciatic nerve was carefully exposed at a point immediately distal from the gluteus maximus muscle. The nerve had been crushed for 30 sec using hemostatic forceps. A crush lesion was performed on the same site (mid-thigh) in both vehicle treated group and experimental treated group.

2.3 Experimental Protocol

The experimental protocol was divided in to 2 parts consisting of the determination of effect of nerve crush injury in diabetic rats and the determination of the effect of zein based nanofiber mats loaded with various concentrations of quercetin or various concentrations of tomato extract on the recovery of sciatic nerve following injury in diabetic rats.

The first part was designed to show the effect of nerve crush injury on sciatic function index of diabetic rats. In this part the animals were divided into groups as described following: 1) control group which receive no treatment 2) Diabetic (DM) group, rats in this group were treated with single shot injection of streptozotocin at dose of 50 mg kg^{-1} in 0.1 M citrate buffer, pH 4.5 via intraperitoneal route 3) DM+sham, rats in this group received STZ to induce diabetes mellitus and receive sham operation at the right sciatic nerve 4)DM+nerve crush injury. Then they were assessed sciatic function index (SCI) which provided the information concerning the integration of sensory and motor functions of sciatic nerve every 3 days throughout the 21-day experimental period.

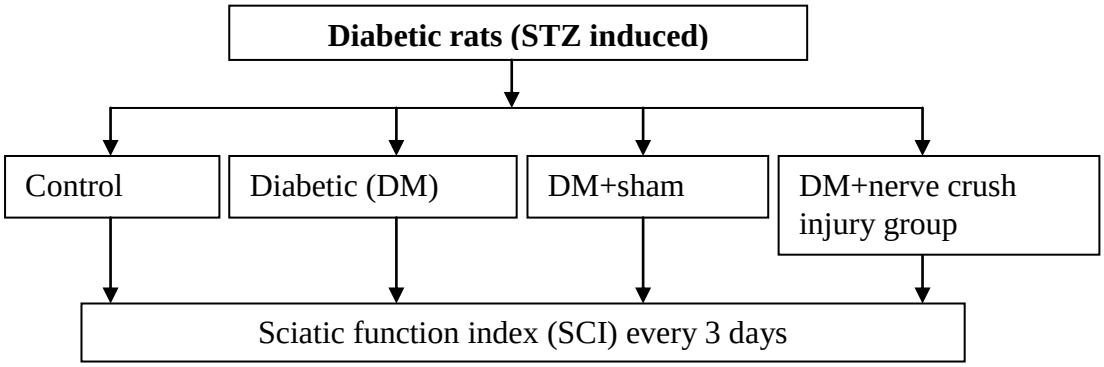


Figure 4-1 Schematic diagram showing experimental protocol for the determination of the effect of nerve crush injury on sciatic function index of diabetic rats

The second part was designed to determine the effect of zein based nanofiber mats loaded with various concentrations of quercetin or various concentrations of tomato extract on the recovery of sciatic nerve following injury in diabetic rats. The first subpart was focused on the effect of zein based nanofiber mats loaded with various concentrations of quercetin on the recovery of sciatic nerve following injury in diabetic rats. The diabetic rats which showed the blood sugar level ≥250 $mg.dL^{-1}$ were divided in to various groups; 1) DM+crush injury and received no treatment 2) DM+crush injury +zein based nanofiber, diabetic rats in this group were subjected to crush injury and zein based nanofiber mats treatment 3) DM+crush injury+5%quercetin, diabetic rats in this group were exposed to crush injury and subjected to 5%quercetin loaded zein based nanofiber 4) DM+crush injury+10%quercetin, diabetic rats in this group were exposed to crush injury and subjected to 10%quercetin loaded zein based nanofiber 5) DM+crush injury+15%quercetin, diabetic rats in this group were exposed to crush injury and subjected to 15%quercetin loaded zein based nanofiber

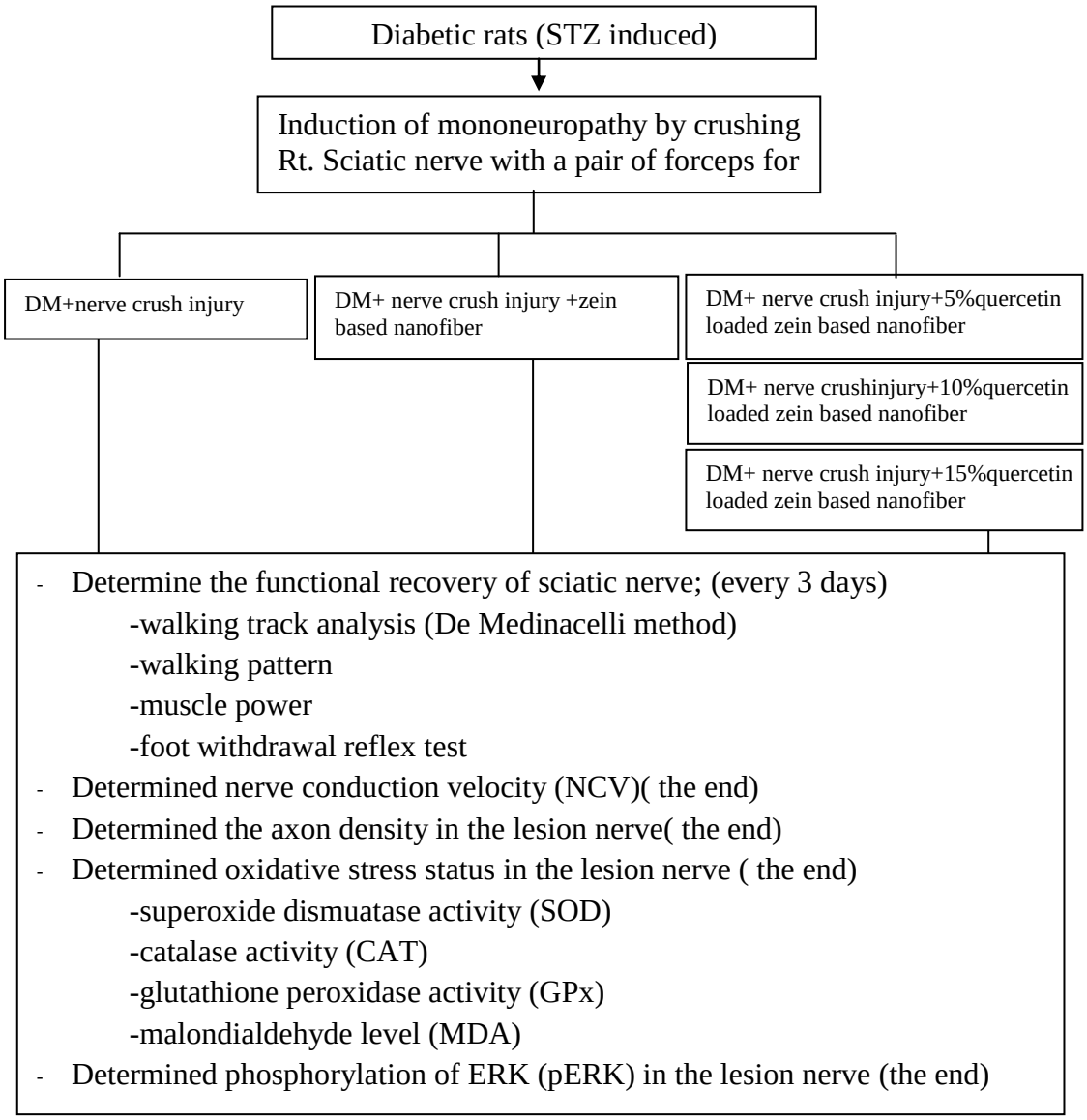


Figure 4-2 Schematic diagram showing experimental protocol for the determination of the effect of zein based nanofiber mats loaded with various concentrations of quercetin in diabetic nerve crush injury

The second subpart was designed to determine the effect of zein based nanofiber mats loaded with various concentrations of tomato extract on the recovery of sciatic nerve following injury in diabetic rats. The animals were divided in to various groups as similar as the first subpart except that diabetic rats in group 3-group5 were exposed to crush injury and subjected to various concentration of tomato extract ranging from 5%,10% and 15%.

All diabetic rats were subjected to the assigned treatment for 21 days. They were assessed the functional recovery of sciatic nerve via walking track analysis (De Medinacelli method), walking pattern assessment, foot withdrawal reflex test and nerve conduction velocity assessment every 3 days throughout the study period. At the end of the study period, they were determined nerve conduction velocity (NCV). The lesion nerve was isolated and determined the axon density, oxidative stress status including the activities of superoxide dismuatase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the level of malondialdehyde (MDA) in the lesioned nerve.

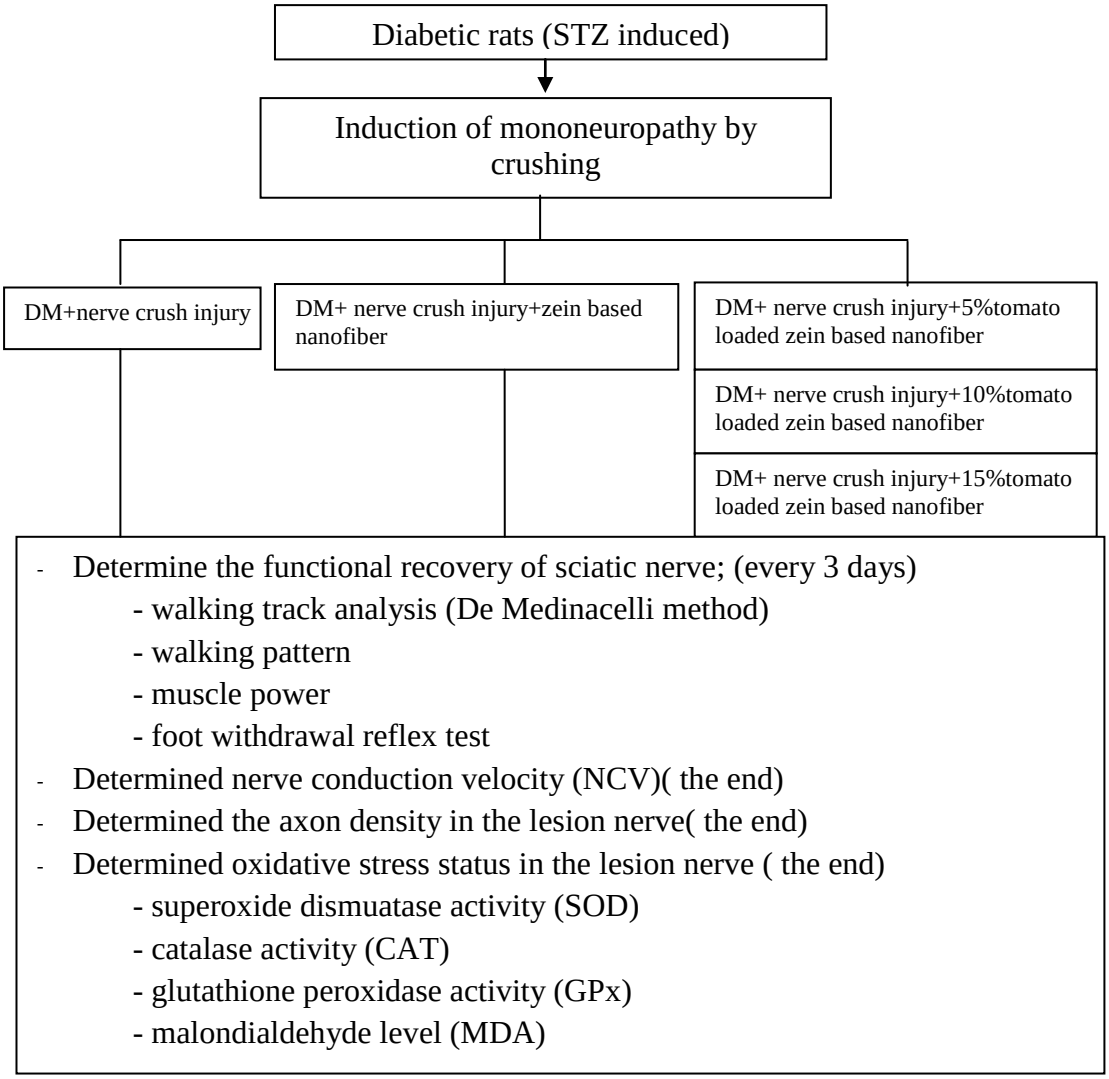
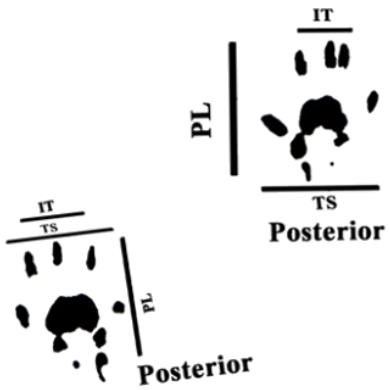


Figure 4-3 Schematic diagram showing experimental protocol for the determination of the effect of zein based nanofiber mats loaded with various concentrations of tomato extract in diabetic nerve crush injury

2.4 Determination of Motor Function Using Walking Track Test (De Medinaceli Method)

Hind feet of animals were dipped in Indian ink and they were placed in to the wooden box 8.2 x 42 cm in dimension which served as a walking track. They were allowed to walk down the track upon a strip of white paper. The prints left by the ink were allowed to dry and then analyzed sciatic function index (SFI) according to the following equation every 3 days throughout the experimental period.

SFI= (-38.3 x PLF) + (109.5 x TSF) + (13.3 x ITF) - 8.8



PLF = (EPL-NPL)/NPL
TSF = (ETS-NTS)/NTS
ITF = (EIT-NIT)/NIT
PL = Print length
TS = Toe spread
IT = Intermediary toe spread
E = Experimental foot
N = Non-operated foot

Figure 4-4 Walking Track Test (De medinacelli method). The equation was used for calculating sciatic function index. The SFI are regarded as normal when they are within the range of -10 to 10%. (Jeroen et al., 2000; Christina et al., 2004)

All measurements were performed manually in blinded fashion and recorded to the nearest millimeter.

2.5 Determination of Motor Function Using Walking Pattern

To assess the walking pattern, all animals in each group were transferred to smooth ground and evaluated walking, standing and balance. The walking pattern was graded as score as following;

- Normal (+4): normal gait (normal standing and walking, no ataxia during walking).
- Mild abnormal (+3): ataxia with wide base gait (animal can stand but ataxia and abduction of both legs were presented).
- Moderate abnormal (+2): ataxia with unstable gait (collapse, ataxia and abduction of both legs were presented sometime during standing).
- Severe abnormal (+1): truncal ataxia (ataxia until the animal cannot walk).

2.6 Determination of Motor Function Muscle Power

The muscle power of the animal was determined using the ability of successfully walking or climbing on the incline area which has been set up at various degrees 0, 90, 120 and 180 as index. Each animal was trained to walk on the incline before testing the muscle power scores angle for 1 week. The amount of time that the animal was still able to walk on the incline was used as parameter for muscle power as described following;

0 = ability to climb at the angle of 90 degree less than 15 seconds.

1 = ability to climb at the angle of 120 degree less than 10 seconds but can climb at the angle of 90 more than 15 seconds.

2 = ability to climb at the angle of 180 degree less than 5 seconds but can climb at the angle of 120 degree more than 10 seconds.

3 = ability to climb at the angle of 180 degree more than 5 seconds.

2.7 Determination of Sensory Function in Respond to Temperature Stimuli by Using

Hot Plate Test

The rats were gently dropped into a plastic box with a metal floor that was preheated to a certain temperature (hot plate) (Liu et al., 2010). The time duration between the initiation of the hot plate exposure and the time which the paw was raised from the floor was recorded and considered as paw withdrawal latency. Each animal

was measured 5 times per session, with the time interval 5 min between tests. The average paw withdrawal latency was calculated and used as index. Minimal and maximal cut offs were assigned at 0.5 to 20 seconds, respectively.

2.8 Determination of sciatic Nerve Conduction by Nerve Conduction Velocity (NCV)

Nerve conduction velocity was assessed at a 21-day intervention period by applying the electrical stimulation at the right sciatic nerve and the response latency required for eliciting the contraction of calf muscles that supplied by sciatic nerve. In brief, the animals were anesthetized with pentobarbital 50 mg/kg BW via intra peritoneal (i.p.) route. Then, an incision was made at the back of the thigh and the sciatic nerve is carefully exposed and cleaned at a point immediately distal to the gluteus maximus muscle. Gastrocnemius muscle was tied to a string by which the muscle was held at a fixed length on the force transducer. The muscle was stimulated via sciatic nerve and the intensities of stimuli were gradually increased until the maximum contraction was observed. The supramaximum intensity of electrical stimulus was applied at stimulus electrode and then recorded the response. The stimulating electrode was moved distally 10 mm away from the previous point. The latencies of muscle contraction were recorded. The nerve conduction velocity was calculated by using the distance between the stimulating and recording electrodes divided by the latency changes after changing location of stimulating electrode.

NCV = 10 mm/ (1st Latency (ms)-2nd Latency (ms)

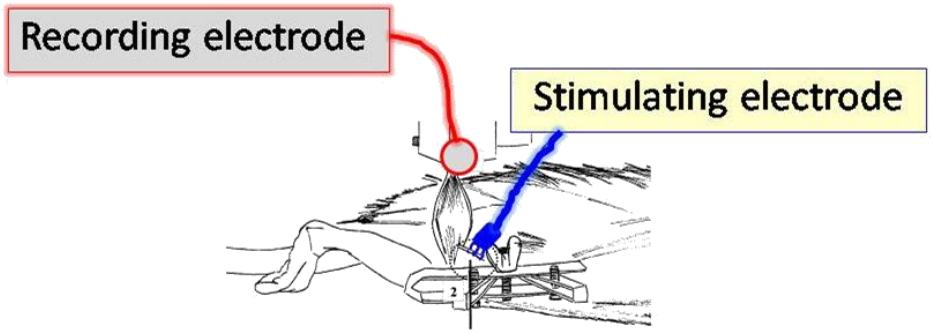


Figure 4-5 The motor nerve conduction velocity study

2.9 Determination of axonal density

At the end of the experiment, all animals were sacrificed and the lesion nerves were collected for histological study. In brief, tissues were embedded in paraffin and cut at 5 µm thick. The sections were stained with toluidine blue. The sections were analyzed by light microscopy. Axonal density was determined by using the program of Image Pro-plus 5.1.

2.10 Determination of oxidative stress markers

After being sacrifice at the end of study, sciatic nerve was isolated. The homogenate of right sciatic nerve was prepared in 1 ml of 0.1 M phosphate buffer, pH 7.4. The obtained nerve homogenate was adjusted to 10 % w/v and centrifuged at 10,000 g, 4°C for 1 hour. The supernatant was harvested and determined the oxidative stress markers including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

2.10.1 Determination of malondialdehyde (MDA)

Malondialdehye (MDA) level was measured by using thiobarbituric acid reacting substances (TBARS) assay. In brief, 100 µl of sample was mixed with the solution containing 100 µl of 8.1% (w/v) sodium dodecyl sulphate, 750 µl 20% (v/v) acetic acid (pH 3.5), and 750 µl of 0.8% thiobarbituric acid (TBA). The solution was heated in a water bath at 95°C for one hour and cooled immediately under running tap water. Then, 500 µl of chilled water and 2500 µl of butanol and pyridine [15:1 v/v] were added into each tube and mixed thoroughly with vortex. Then, the solution was centrifuged at 800 x g for 20 minutes. The upper layer was separated and measured absorbance at 532 nm. 1,3,3-tetra ethoxy propane (TEP) was used as the reference (Ohkawa *et al.*, 1979). The level of MDA was expressed as U/mg.protein.

2.10.2 Determination of superoxide dismutase (SOD)

The determination of SOD activity was performed by using nitrobluetetrazolium (NBT) reduction assay. In this assay, the xanthine - xanthine oxidase system was used as a superoxide generator. In brief, the reaction mixture contained 20 µl of sample and 200 µl of reaction mixture consisting of 57 mM phosphate buffer solution (KH2PO4), 0.1 mM EDTA, 10 mM cytochrome C solution and 50 µM of xanthine solution and 20 µl of xanthine oxidase solution (0.90mU/ml) were prepared at 25ºC.The optical density was determined at 415 nm. A system devoid of enzyme was served as

the control and three parallel experiments were conducted (Sun *et al.*, 1988). SOD activity was expressed as U/mg.protein.

2.10.3 Determination of catalase (CAT)

Nerve catalase activity was evaluated by determining the ability of the enzyme to break down H_2O_2. In brief, 10 µl of sample was mixed with the reaction mixture which contained 50 µl of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0), 25 µl of H2SO4 and 150 µl of KMnO4. After mixing thoroughly, the optical density was measured at 490 nm. A system devoid of the substrate (hydrogen peroxide) was served as the control. The difference in absorbance per unit time was expressed as the activity. An amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25º is regarded as one unit (Goth, 1991).The value of CAT activity was expressed as U/mg.protein.

2.10.4 Determination of glutathione peroxidase (GPx)

This assay was carried out by using the glutathione recycling method by using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and glutathione reductase. According to this method, the reaction between DTNB and GSH gave rise to the generation of 2-nitro-5-thiobenzoic acid and GSSG. Since 2-nitro-5-thiobenzoic acid was a yellow colored product, GSH concentration could be determined by measuring absorbance at 412 nm. In brief, a mixture containing a 20 µl of sample and the reaction mixture consisting of 10 µl of dithiothreitol (DTT) in 6.67 mM potassium phosphate buffer (pH 7), 100 µl of sodium azide in 6.67 mM potassium phosphate buffer (pH 7), 10 µl of glutathione solution and 100 µl of hydrogen peroxide, was mixed thoroughly and incubated at room temperature for 5-10 minutes. Then, 10 µl of DTNB (5,5-dithiobis-2-nitrobenzoic acid) was added and the optical density at 412 nm was recorded at 25 ºC over a period of 5 min. (Rotruck et al., 1973). GPx activity was expressed as U/mg.protein.

2.11 Western Blot Analysis

The lesion sciatic nerves were isolated and prepared as homogenate by using ice cold homogenization buffer [RIPA buffer with protease inhibitor cocktail]. The nerve homogenate was centrifuged at 14,000 g for 30 min, and the supernatant was collected. Protein concentration was determined using NANO drop Spectrophotometers. Equal amount of protein (70 µg) was separated by SDS-PAGE (10% SDS-polyacrylamide gel electrophoresis) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-

Rad Laboratories, Hercules, CA). After transferring to membrane, prevention of non-specific binding on the membrane was achieved by incubating with 10% dried skim milk in Tween buffer (0.05% Tween20, Sigma) (Agthong et al, 2006) for 1 h at room temperature, and incubated overnight either with phospho-ERK1/2 (1:1,000, Cell Signaling Cell Signaling Technology, Inc., Boston, MA, USA) or with total ERK1/2 (1:1,000, Cell Signaling Cell Signaling Technology, Inc., Boston, MA, USA). After the incubation, the membrane was subjected to several washing steps. HRP-linked secondary antibody (1:1,000) was incubated with the membrane for 1 hr at room temperature and signals were visualized by chemiluminescence using a ECL kit (Pierce, ThermoScientific). The intensity of gray scale image was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Data were presented as the intensity of ERK or the ratio of pERK/ERK. (Kiguchi N et al, 2009)

2.12 Statistical Analysis

Data were shown as mean±SEM. One way analysis of variance (ANOVA) and Post-hoc Tukey test were used as appropriated and p-value<0.05 was considered as statistically significant.

3. Results

3.1 Effect of the nerve crush injury on sciatic function index (SFI) of diabetic rats

Table 4-1 showed the effect of nerve crush injury in diabetic rats. It was found that neither diabetic rats nor diabetic rats which subjected to sham operation showed the significant difference of SFI when compared to control rats. However, diabetic rats which subjected to crush injury showed the impairment of SFI throughout the 21-day study period (normal value of SFI was varied between -10 to +10).

3.2 Effect of quercetin loaded zein based nanofiber on the functional recovery of sciatic nerve of diabetic rats

Table 4-2 showed that diabetic rats which subjected to crush injury and received zein based nanofiber mat showed the significant improved SFI at 9-day study period and at 18-day study period (P-value<.001 and .05 respectively; compared to DM+crush injury). At 9 day-study period, diabetic rats which exposed to crush injury and zein based nanofiber mats loaded with 5% and 10%quercetin significantly

improved SFI (P-value<.01 and .05 respectively; compared to DM+crush injury+zein based nanofiber mat).It was found that diabetic rats which exposed to crush injury and zein based nanofiber mats loaded with 5%, 10% and 15%quercetin showed the improved SFI at 15 day-study period (P-value<.05,.01 and .05 respectively, compared to DM+crush injury+zein based nanofiber mat). In addition, the current data also showed that diabetic rats which subjected to crush injury and quercetin loaded nanofiber mat at 5%,10% and 15% showed the SFI within normal range (-10 to +10) at the end of experiment whereas the other groups still failed to gain full recovery.

The walking pattern was also assessed and data were shown in table 4-3. Diabetic rats subjected to crush injury and zein based nanofiber mat significantly impaired walking pattern (P-value<.001; compared to DM+crush injury) at 6 day-study period. Interestingly, the walking pattern of diabetic rats subjected to crush injury and15quercetin loaded zein based nanofiber significantly improved at 1,3 and 9 days of treatment (P-value<.01, .01 and .05 respectively; compared to DM+crush injury+zein based nanofiber mat). Within 12 days of treatment, diabetic rats which subjected to crush injury and received zein based nanofiber mat or zein based nanofiber loaded with quercetin at concentrations of 5%, 10% and 15% showed the normal value of walking pattern as shown in table 4-3.

The animals were also assessed muscle power and results were shown in table 4-4. Diabetic rats which exposed to crush injury and received zein based nanofiber mat treatment significantly improved muscle power at day 3, day 6 and day 9 (P-value<.05, .01 and .01 respectively; compared to DM+crush injury).The diabetic rats which exposed to crush injury and received 5%quercetin loaded zein based nanofiber showed the significant improvement of muscle power at 1 day of treatment (P-value<.001; compared to DM+crush injury+zein based nanofiber mat). No other significant changes were observed.

On the basis that sciatic nerve was the mixed nerve and performed both motor and sensory functions, the foot withdrawal reflex latency of sciatic nerve in respond to mechanical stimuli was also assessed using hot plate test and results were shown in table 4-5. It was found that diabetic rats subjected to crush injury and zein based nanofiber mat significantly decreased foot withdrawal latency at 6-day and 9-day study

periods (P-value<.01 all; compared to DM+crush injury). At 3-day study period zein based nanofiber mat loaded with 5%, 10% and 15% significantly improved withdrawal latency (P-value<.01, .05 and .001 respectively; compared to DM+crush injury+zein based nanofiber mat).

The effect of quercetin loaded zein based nanofiber on nerve conduction velocity was also assessed. Figure 4-6 showed that diabetic rats subjected to crush injury and zein based nanofiber mat failed to show the improved nerve conduction velocity. However, zein based loaded nanofiber mats loaded with quercetin 5%, 10% and 15% improved the nerve conduction velocity of diabetic rats which subjected to crush injury (P-value <.05 all; compared to DM+crush injury+zein based nanofiber mat). In addition, the effect of quercetin loaded zein based nanofiber on axon density was also determined. Figure 4-7 showed that zein based nanofiber mat failed to increase the axon density in diabetic rats which exposed to crush injury. Diabetic rats subjected to crush injury and treated with zein based nanofiber loaded with 5% and 10% quercetin showed the significant increase in axon density (P-value<.01 and .001 respectively; compared to DM+crush injury+zein based nanofiber mat).

Table 4-6 showed the effect of quercetin loaded zein based nanofiber on the oxidative stress markers including MDA level and the activities of SOD, CAT and GPx in the lesion nerve. Diabetic rats which exposed to crush injury and received zein based naofiber mat failed to show the significant changes of all parameters mentioned earlier. Interestingly, diabetic rats which subjected to crush injury and received all concentrations of quercetin loaded zein based nanofiber significantly decreased MDA level (P-value<.01, .05 and .01 respectively; compared to DM+crush injury+zein based nanofiber mat) but increased the activities of GPx (P-value<.05 all; compared to DM+crush injury+zein based nanofiber mat) and CAT (P-value<.05, .05 and .01 respectively; compared to DM+crush injury+zein based nanofiber mat). The elevation of SOD activity was observed only in diabetic rats which subjected to crush injury and received zein based nanofiber mats loaded with 5% and 10% quercetin (P-value<.05 and .001 respectively; compared to DM+crush injury+zein based nanofiber mat). Since neurite extension, and the plasticity of nervous system was associated with the function of growth factor and ERK or MAPK, the effect of quercetin loaded zein

based nanofiber on the alteration of ERK in the lesion nerve. Figure 4-8 showed that diabetic rats with sciatic nerve lesion which received 5%, quercetin-loaded zein based nanofiber mats showed the significant elevation pERK1/2 level in the lesion nerve both at 3 days and 7 days of treatment (P-value<.01 and .05 respectively; compared to DM+crush injury+zein based nanofiber mat) while diabetic rats with sciatic nerve lesion which received 10%, quercetin-loaded zein based nanofiber mats showed the significant elevation pERK1/2 level in the lesion nerve only at 6 days of treatment (P-value<.01; compared to DM+crush injury+zein based nanofiber mat)

Table 4-1 Effect of nerve crush injury and diabetes mellitus on sciatic function index evaluated by walking track analysis. (Normal SFI is between -10 and +10) (n=8/group)

	Sciatic Function Nerve Index (SFI) at various intervention periods								
Groups	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
Control	-4.87±3.20	-7.43±1.60	0.57±3.20	-5.48±5.80	-1.58±3.20	-2.40±3.20	-1.58±3.20	-1.58±-3.19	0.64±3.23
Diabetic(DM)	-3.33±2.90	-7.71±1.26	-0.01±2.56	-4.54±4.40	-1.84±2.90	-1.84±2.90	-1.84±2.90	-0.93±-2.59	-1.84±2.91
DM+sham	0.04±2.58	10.51±6.31	-8.96±3.26	-9.08±2.43	-2.32±2.75	-6.28±3.15	-5.19±1.87	-3.16±-2.68	-3.45±2.72
DM+nerve crush injury	-8.43±1.20	-93.3±0.00 ***	-93.3±0.00 ***	-93.3±0.00 ***	-93.3±0.00 ***	-89.57±2.70 ***	-62.84±8.80 ***	-57.50±-2.74 ***	-37.76±6.65 ***

***P-value<0.001; compared to diabetic (DM) group.

Table 4-2 Effect of quercetin loaded zein based nanofiber on sciatic nerve function index of diabetic rats of various treatment groups evaluated by walking track analysis (n=8/group) The SFI are regarded as normal when they are within the range of -10 to 10%

Sciatic Function Nerve Index (SFI) at various intervention periods									
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	-8.42±1.23	-93.3±0.00	-93.3±0.00	-93.3±0.00	-93.30±0.00	-70.01±2.70	-62.84±8.78	-57.50±2.74	-37.75±6.65
DM+ nerve crush injury +zein based nanofiber	-3.33±6.64	-93.3±0.00	-93.3±0.01	-93.3±0.01	-67.53±5.86 aaa	-63.27±9.46 a	-53.31±6.32	-44.94±4.62	-16.87±1.58 aaa
DM+ nerve crush injury + 5%quercetin loaded zein based nanofiber	-4.68±7.14	-93.3±0.00	-93.3±0.02	-93.3±0.02	-56.30±2.07 aaa##	-53.66±9.39 aa	-31.49±6.02 aaa##	-23.53±4.22 a#	-9.11±4.66 aaa
DM+ nerve crush injury +10%quercetin loaded zein based nanofiber	-4.94±3.90	-93.3±0.00	-93.3±0.03	-93.3±0.03	-61.69±2.98 aaa	-56.59±13.17 aa	-26.59±3.61 aaa##	-24.78±3.55 aaa###	-9.16±1.18 aaa##
DM+ nerve crush injury + 15%quercetin loaded zein based nanofiber	-8.73±1.13	-93.3±0.00	-93.3±0.04	-93.3±0.04	-93.30±0.00	-78.59±9.11	-30.04±7.64 aaa##	-26.18±6.45 aaa###	-8.36±1.26 aaa###

[a, aa, aaa] p-value<.05, .01 and .001 respectively; compared to DM+ nerve crush injury group.

[#, ##] p-value<.05 and .01 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.

Table 4-3 Effect of quercetin loaded zein based nanofiber mat on walking pattern of diabetic rats with crush injury (n=8/group)

	Walking pattern (grade)								
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	4.00±0.00	1.25±0.16	1.25±0.16	1.88±0.12	2.56±0.17	2.75±0.16	3.13±0.23	3.38±0.18	3.38±0.18
DM+ nerve crush injury +zein based nanofiber	4.00±0.00	1.13±0.13	2.00±0.26	2.88±0.15 aaa	2.62±0.18	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
DM+ nerve crush injury + 5%quercetin loaded zein based nanofiber	4.00±0.00	1.13±0.13	1.75±0.16	2.69±0.16	2.94±0.14	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
DM+ nerve crush injury +10%quercetin loaded zein based nanofiber	4.00±0.00	1.00±0.00	1.69±0.21	2.38±0.32	2.50±0.26	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
DM+ nerve crush injury + 15%quercetin loaded zein based nanofiber	4.00±0.00	1.75±0.25 aaa##	2.88±0.23 aaa##	2.75±0.16 aaa	3.44±0.17 aa#	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00

aa, aaa P-value< .01 and .001 respectively; compared to DM+ nerve crush injury group.

#, ## P-value<.05 and .01 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.

Table 4-4 Effect of quercetin loaded zein based nanofiber mat on muscle power of diabetic rats with crush injury (n=8/group)

	Muscle power (score)								
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	3.00±0.00	0.00±0.00	0.13±0.13	0.63±0.18	1.13±0.23	2.13±0.22	3.00±0.00	3.00±0.00	3.00±0.00
DM+ nerve crush injury +zein based nanofiber	3.00±0.00	0.00±0.01	1.00±0.27 a	1.63±0.18 aa	2.00±0.00 aa	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
DM+ nerve crush injury + 5%quercetin loaded zein based nanofiber	3.00±0.00	0.63±0.18 aaa###	1.00±0.19 a	1.75±0.16 aa	1.63±0.38	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
DM+ nerve crush injury +10%quercetin loaded zein based nanofiber	3.00±0.00	0.25±0.16	0.88±0.30[a]	1.75±0.16 aa	2.00±0.00 aa	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
DM+ nerve crush injury + 15%quercetin loaded zein based nanofiber	3.00±0.00	0.13±0.13	0.63±0.18	1.75±0.17 aa	2.50±0.19 aaa	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00

[a, aa, aaa] P-value< .05, .01 and .001 respectively; compared to DM+ nerve crush injury group.

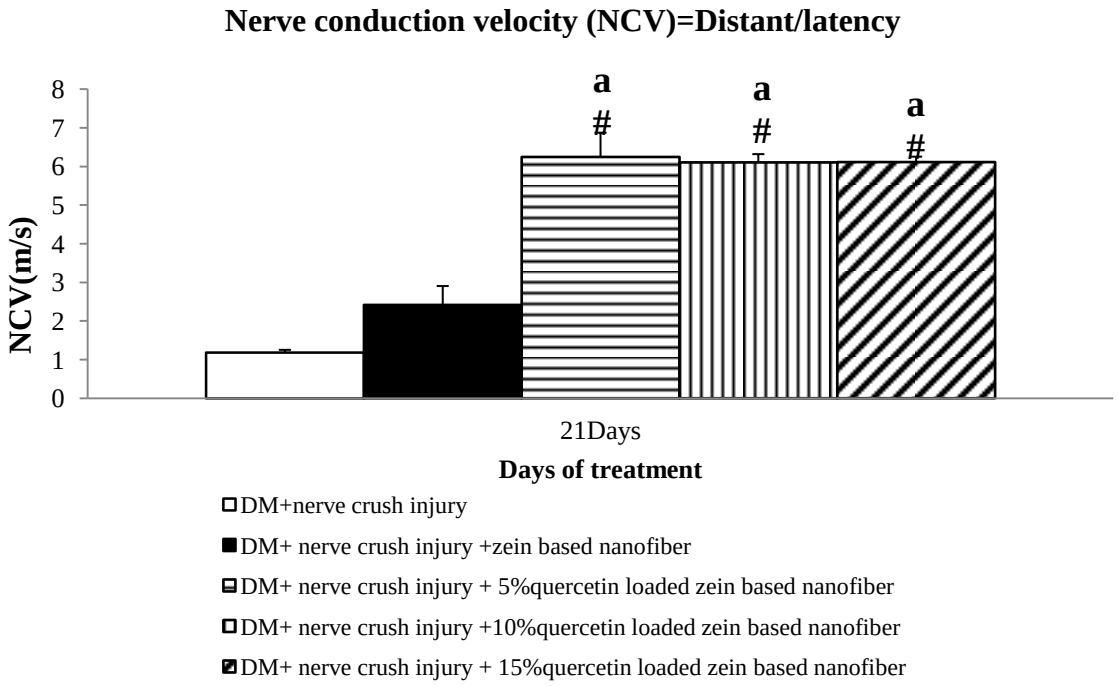
[###] P-value<.001; compared to DM+ nerve crush injury +zein based nanofiber group.

Table 4-5 Effect of quercetin loaded zein based nanofiber mat on paw withdrawal latency of diabetic rats with crush injury (n=8/group)

	Paw withdrawal latency (s.)								
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	2.40±0.58	3.75±0.56	5.09±0.79	4.06±0.83	2.53±0.39	1.75±0.13	1.93±0.09	1.31±0.07	1.25±0.06
DM+ nerve crush injury +zein based nanofiber	2.12±0.18	3.04±0.30	5.18±0.41	2.31±0.25 aa	1.78±0.17 aa	1.62±0.14	1.62±0.11	1.12±0.06	1.12±0.06
DM+ nerve crush injury + 5%quercetin loaded zein based nanofiber	1.87±0.10	2.76±0.23 aaa##	2.40±0.24 aa##	1.94±0.19 aaa	2.04±0.29 a	1.87±0.15	1.87±0.16	1.06±0.04	1.04±0.04
DM+ nerve crush injury +10%quercetin loaded zein based nanofiber	1.90±0.14	3.15±0.38 aaa	3.34±0.68 a#	1.59±0.13 aaa	1.81±0.14 aa	1.56±0.10	1.90±0.15	1.03±0.03	1.12±0.06
DM+ nerve crush injury + 15%quercetin loaded zein based nanofiber	1.97±0.00	3.53±0.29 aa	2.19±0.21 aaa###	2.57±0.22 a	1.96±0.15	1.83±0.25	2.12±0.34	1.53±0.05	1.12±0.06

aa, aaa P-value< .01 and .001 respectively; compared to DM+ nerve crush injury group.

#, ## P-value<.05 and .01 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.



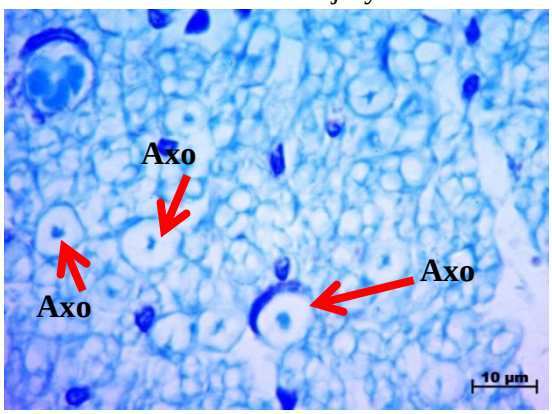
[a] P-value< .05; compared to DM+ nerve crush injury group.

[#] P-value<.05; compared to DM+ nerve crush injury +zein based nanofiber group.

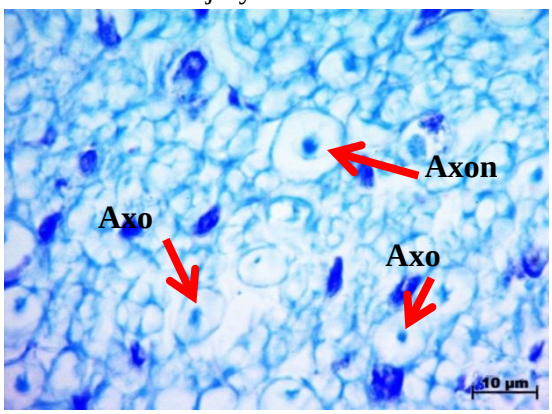
Figure 4-6 Effect of quercetin loaded zein based nanofiber mat on nerve conduction velocity (NCV) of diabetic rats with crush injury (n=8/group)

(A)

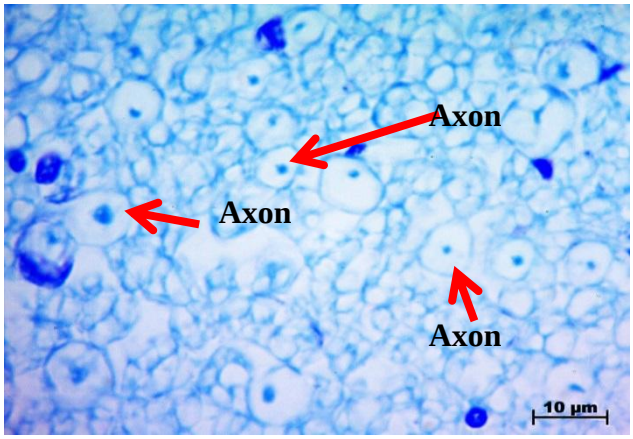
DM+ nerve crush injury



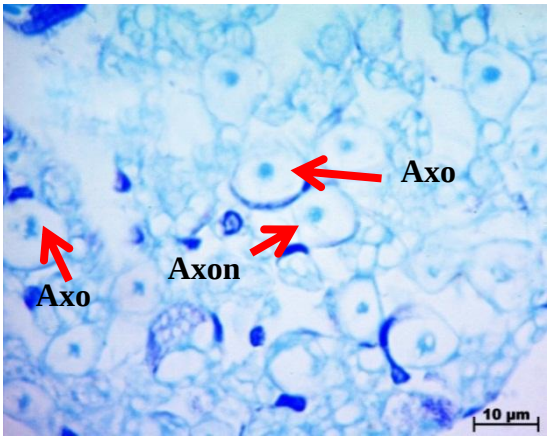
DM+ nerve crush injury +zein based nanofiber



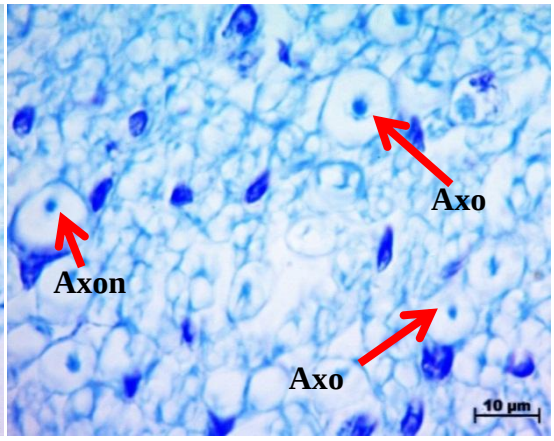
DM+ nerve crush injury + 5%quercetin loaded zein based nanofiber



DM+ nerve crush injury + 10%quercetin loaded zein based nanofiber



DM+ nerve crush injury + 15%quercetin loaded zein based nanofiber



(B)

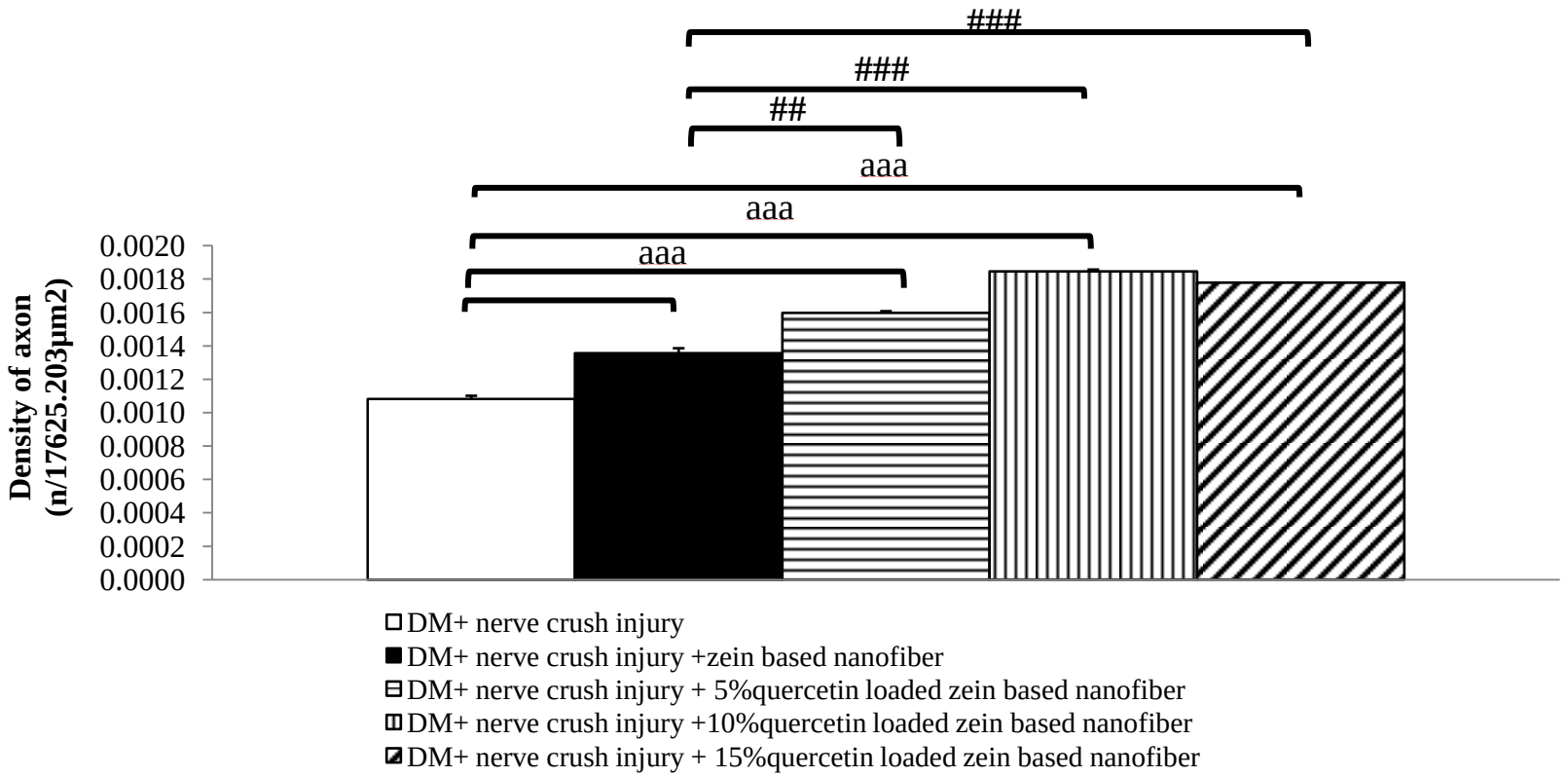


Figure 4-7 Effect of quercetin loaded zein based nanofiber mat on axon density of sciatic nerve of diabetic rats with crush injury.

(A) Photographs of the lesion nerve stained with toluidine blue at 100X magnification

(B) Bar graph showing the density of axon in the lesion nerve of various treatments groups. (n=8/group)

[aaa] P-value< .001; compared to DM+ nerve crush injury group.

[##, ###] P-value<.05 and .001 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.

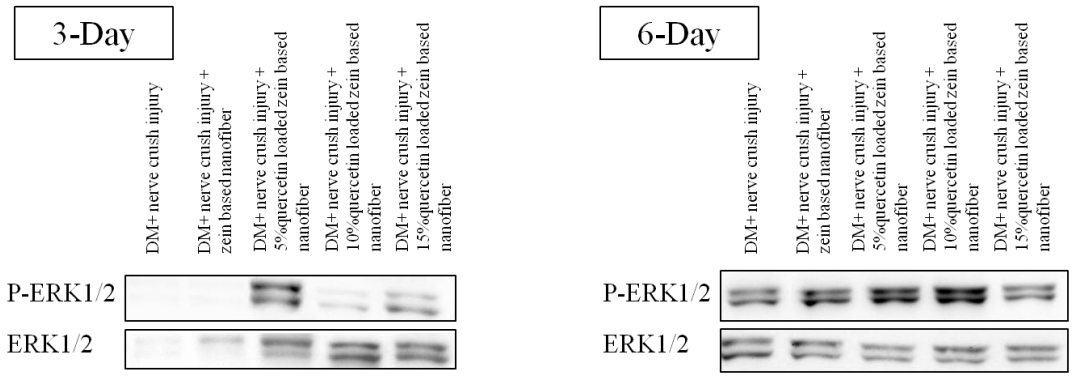
Table 4-6 Effect of quercetin loaded zein based nanofiber mat on oxidative stress markers of sciatic nerve of diabetic rats with crush injury. (n=8/group)

Group	MDA level (nmol/min.g.protein)	SOD activity (u/mg. protein)	CAT activity (u/mg. protein)	GPx.activity (u/mg. protein)
DM+nerve crush injury	0.02±0.005	3.22±0.023	9.87±1.83	9.69±1.17
DM+ nerve crush injury +zein based nanofiber	0.02±0.004	2.75±0.32	7.72±1.24	6.62±1.12
DM+ nerve crush injury + 5%quercetin loaded zein based nanofiber	0.01±0.002 aa##	11.77±2.47 #	21.55±5.23 a#	24.51±4.12 aaa#
DM+ nerve crush injury +10%quercetin loaded zein based nanofiber	0.01±0.001 a#	23.80±2.82 aaa###	23.82±5.56 a#	24.16±5.09 aa#
DM+ nerve crush injury + 15%quercetin loaded zein based nanofiber	0.00±0.000	5.00±0.94	19.74±3.16 a##	18.22±3.06 a#

[a, aa, aaa] P-value< .05, .01 and .001 respectively; compared to DM+nerve crush injury group.

[#, ##, ###] P-value<.05, .01 and .001 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.

(A)



(B)

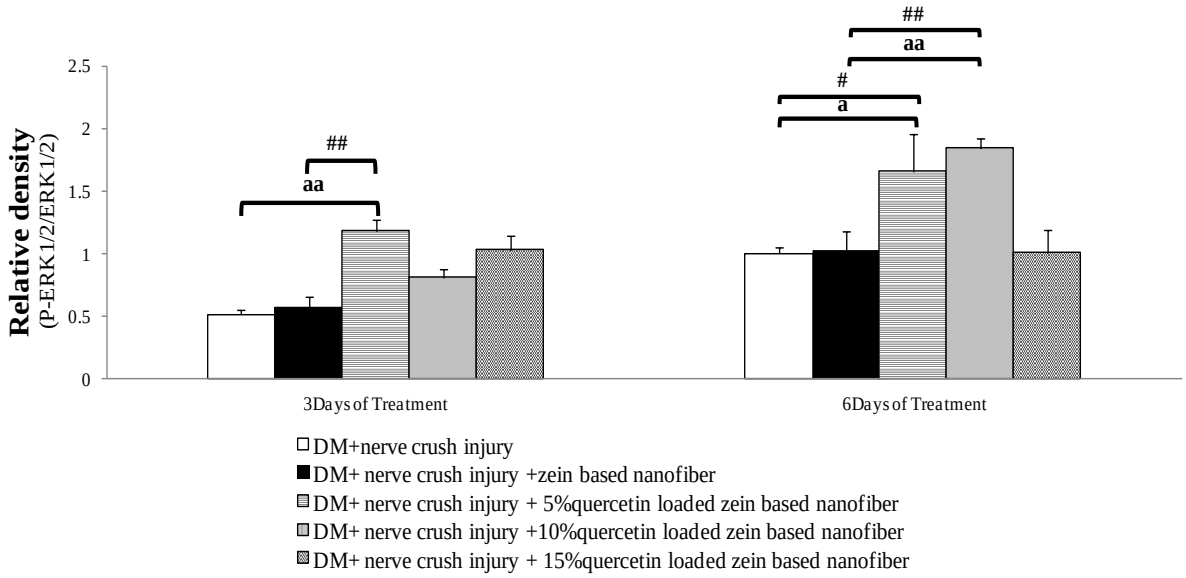


Figure 4-8 Effect of quercetin loaded zein based nanofiber on the level of ERK1/2 and pERK1/2 in sciatic lesion nerve of diabetic rats

(A) The expression of ERK1/2 and pERK1/2 in the lesion nerve of various treatments groups at 3 and 6days of treatment

(B) Bar graph showing the density of level of ERK1/2 and pERK1/2 in sciatic lesion nerve of various treatments groups. (n=8/group)

[a,aa] P-value<.05 and .01 respectively; compared to DM+ nerve crush injury group.

[#, ##] P-value<.05 and .01 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.

3.3 The Effect of Tomato Extract Loaded Zein Based Nanofiber on the Functional Recovery of Sciatic Nerve of Diabetic Rats

Table 4-7 showed that zein based nanofiber mat significantly enhanced SFI of diabetic rats which exposed to crush injury at 9, 12, 18 and 21 days of treatment (P-value<.001, .05,.05 and .05 respectively; compared to diabetic rats with crush injury). Only diabetic rats which received crush injury and zein based nanofiber mat loaded with 5% tomato extract significantly improved the SFI value (P-value< .05, .01 and .001 respectively; compared to diabetic rats with crush injury which received zein based nanofiber mat). In addition, diabetic rats which received crush injury and zein based nanofiber mat loaded with 5% tomato extract also showed the normal value of SFI (-10 to +10) while no other groups showed the normal SFI value.

The effect of zein based nanofiber mat loaded with tomato extract on walking pattern was shown in Table 4-8. Diabetic rats which received crush injury and zein based nanofiber mat showed the significant improvement of walking pattern at 6 day-study period (P-value<.001; compared to diabetic rats with crush injury). Diabetic rats with crush injury which obtained 5%tomato extract significantly improved walking pattern at 1 day-study period (P-value<.05; compared to diabetic rats with crush injury which received zein based nanofiber mat) whereas diabetic rats with crush injury which received 10%tomato extract produced the significant improvement of walking pattern at 1, 6 and 9 days of treatment (P-value<.001, .05 and .05 respectively; compared to diabetic rats with crush injury which received zein based nanofiber mat). Although no other significant changes were observed, the walking pattern s of diabetic rats with crush injury which received either zein based nanofiber mat or zein based nanofiber mat loaded with tomato extract were fully recovery at 12 days of treatment.

The effect of tomato extract loaded zein based nanofiber mat on muscle power of diabetic rats with crush injury was also investigated and results were shown in Table 4-9. Zein based nanofiber mat failed to produce significant change in muscle power of diabetic rats which received crush injury. Both diabetic rats with crush injury which received 5% tomato loaded zein based nanofiber mat and diabetic rats with crush injury which received10%tomato loaded zein based nanofiber mats

produced the significant improvement of walking pattern at 9 days of intervention (P-value<.05 all; compared to diabetic rats with crush injury which received zein based nanofiber mat).

Table 4-10 showed the effect of tomato extract loaded zein based nanofiber mat on paw withdrawal latency in hot plate test of diabetic rats with crush injury. It was found that diabetic rats with crush injury which obtained zein based nanofiber mat improved the increased foot withdrawal latency induced by diabetic condition and crush injury (P-value<.01 all; compared to diabetic rats with crush injury). The significant improvement of paw withdrawal latency induced by tomato extract was observed only in diabetic rats with crush injury which received 5%tomato extract loaded zein based nanofiber mat at 3-day study period (P-value<.05; compared to diabetic rats with crush injury which received zein based nanofiber mat). No significant changes were observed in other groups.

According to Figure 4-9, it was found that diabetic rats plus crush injury and zein based nanofiber mat failed to increase nerve conduction velocity when compared to diabetic rats which received crush injury. Interestingly, diabetic rats with crush injury which received 10%tomato extract loaded zein based nanofiber mat significantly enhanced nerve conduction velocity (P-value<.05 all; compared to diabetic rats with crush injury and compared to diabetic rats with crush injury which received zein based nanofiber mat). Unfortunately no significant changes were observed in other groups.

The effect of tomato extract loaded zein based nanofiber mat on axon density was also explored. Unfortunately no significant changes were observed in any treatment groups as shown in Figure 4-10.

The effect of tomato extract loaded nanofiber mat on oxidative stress markers was shown in Table 4-11. Diabetic rats which received zein based nanofiber mat significantly decreased MDA level (P-value<.05; compared to diabetic rats whch received NSS) but produced no significant changes of SOD,CAT and GPx activities in the lesion nerve. In addition, diabetic rats which received 5% and 15% tomato extract loaded zein based nanofiber significantly increased SOD and GPx (P-value<.01, .05; .01,.05 respectively; compared to diabetic rats which received zein based nanofiber mat) whereas diabetic rats which received 10% tomato extract loaded zein based nanofiber showed the significant increase in SOD, CAT and GPx (P-value<.05 all; compared to diabetic rats which received zein based nanofiber mat).

Table 4-7 Effect of tomato extract loaded zein based nanofiber on sciatic nerve function index of diabetic rats of various treatment groups evaluated by walking track analysis (n=8/group) The SFI are regarded as normal when they are within the range of 10 to 10%

	Sciatic nerve function index (SFI)								
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	-8.42±1.23	-93.3±0.00	-93.3±0.00	-93.3±0.00	-93.3±0.00	-70.01±2.7	-62.84±8.78	-57.50±2.74	-37.75±6.65
DM+ nerve crush injury +zein based nanofiber	-3.33±6.64	-93.3±0.00	-93.3±0.00	-93.3±0.00	-67.53±5.86 aaa	-63.27±9.46 a	-53.31±6.32	-44.94±4.62 a	-16.87±1.58 aaa
DM+ nerve crush injury + 5%tomato loaded zein based nanofiber	-6.65±5.70	-93.3±0.00	-93.3±0.00	-93.3±0.00	-68.25±1.23 aaa	-70.90±8.06	-62.95±4.79	-45.65±5.03 aaa###	-9.09±2.01 aaa##
DM+ nerve crush injury +10%tomato loaded zein based nanofiber	-5.02±4.74	-93.3±0.00	-93.3±0.00	-93.3±0.00	-93.3±0.00	-59.82±6.46 aa	-44.37±4.95 a	-36.56±3.32 aaa###	-13.02±3.79 aaa
DM+ nerve crush injury + 15%tomato loaded zein based nanofiber	-7.08±5.23	-93.3±0.00	-93.3±0.00	-93.3±0.00	-60.59±4.07 aaa	-64.39±6.58 a	-56.25±7.64	-54.94±4.26	-19.35±6.27 aaa

a, aa, aaa p-value<.05, .01 and .001 respectively; compared to DM+ nerve crush injury group.

#,##, ### p-value<.05, .01and .001 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.

Table 4-8 Effect of tomato extract loaded zein based nanofiber mat on walking pattern of diabetic rats with crush injury (n=8/group)

	Walking pattern (grade)								
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	4.00±0.00	1.25±0.16	1.25±0.16	1.88±0.12	2.56±0.17	2.75±0.16	3.13±0.23	3.38±0.18	3.38±0.18
DM+ nerve crush injury +zein based nanofiber	4.00±0.00	1.13±0.13	2.00±0.26	2.88±0.15 aaa	2.62±0.18	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
DM+ nerve crush injury + 5%tomato loaded zein based nanofiber	4.00±0.00	1.63±0.18 aa#	2.57±0.17 aaa	2.56±0.17 aa	2.31±0.36	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
DM+ nerve crush injury +10%tomato loaded zein based nanofiber	4.00±0.00	2.00±0.26 aaa###	2.63±0.41 aaa	3.5±0.18 aaa#	3.38±0.32 a#	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
DM+ nerve crush injury + 15%tomato loaded zein based nanofiber	4.00±0.00	1.38±0.18	2.00±0.26	3.13±0.29 aaa	2.75±0.45	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00

a, aa, aaa P-value<.05, .01 and .001 respectively; compared to DM+ nerve crush injury group.

#, ###P-value<.05 and .001 respectively; compared to DM+ nerve crush injury +zein based nanofiber group.

Table 4-9 Effect of tomato extract loaded zein based nanofiber mat on muscle power of diabetic rats with crush injury (n=8/group)

	Muscle power (score)								
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	3.00±0.00	0.00±0.00	0.13±0.13	0.63±0.18	1.13±0.23	2.13±0.22	3.00±0.00	3.00±0.00	3.00±0.00
DM+ nerve crush injury +zein based nanofiber	3.00±0.00	0.00±0.01	1.00±0.27	1.63±0.18	2.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
DM+ nerve crush injury + 5%tomato loaded zein based nanofiber	3.00±0.00	0.00±0.00	0.38±0.18	1.50±0.19 [a]	2.63±0.18 [aaa#]	3.00±0.01	3.00±0.01	3.00±0.01	3.00±0.01
DM+ nerve crush injury +10%tomato loaded zein based nanofiber	3.00±0.00	0.00±0.01	0.50±0.19	1.13±0.29	1.75±0.25 [a#]	3.00±0.02	3.00±0.02	3.00±0.02	3.00±0.02
DM+ nerve crush injury + 15%tomato loaded zein based nanofiber	3.00±0.00	0.00±0.02	0.50±0.19	1.38±0.46	1.38±0.38	3.00±0.03	3.00±0.03	3.00±0.03	3.00±0.03

[a, aaa] P-value< .05 and .001 respectively; compared to DM+ nerve crush injury group.

[#]P-value<.05; compared to DM+ nerve crush injury +zein based nanofiber group.

Table 4-10 Effect of tomato extract loaded zein based nanofiber mat on paw withdrawal latency of diabetic rats with crush injury (n=8/group)

	Paw withdrawal latency (s.)								
Group	**Baseline**	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**	**18-day**	**21-day**
DM+nerve crush injury	2.40±0.58	3.75±0.56	5.09±0.79	4.06±0.83	2.53±0.39	1.75±0.13	1.93±0.09	1.31±0.07	1.25±0.06
DM+ nerve crush injury +zein based nanofiber	2.12±0.18	3.04±0.30	5.18±0.41	2.31±0.25 aa	1.78±0.17 aa	1.62±0.14	1.62±0.11	1.12±0.06	1.12±0.06
DM+ nerve crush injury + 5%tomato loaded zein based nanofiber	1.70±0.13	3.34±0.40 a	3.34±0.37 a#	2.37±0.20 aa	1.87±0.10	1.59±0.10	1.59±0.20	1.09±0.04	1.08±0.05
DM+ nerve crush injury +10%tomato loaded zein based nanofiber	1.93±0.29	3.31±0.39 a	4.03±0.73	1.90±0.16 aaa	1.94±0.11 a	1.87±0.09	1.81±0.25	1.12±0.04	1.12±0.06
DM+ nerve crush injury +15%tomato loaded zein based nanofiber	2.23±0.28	3.03±0.36 a	4.43±0.36	1.78±0.13 aaa	1.75±0.14 a	2.00±0.17	2.18±0.25	1.09±0.04	1.04±0.04

[a,aa, aaa] P-value< .05, .01 and .001 respectively; compared to DM+ nerve crush injury group.

[#]P-value<.05; compared to DM+ nerve crush injury +zein based nanofiber group.

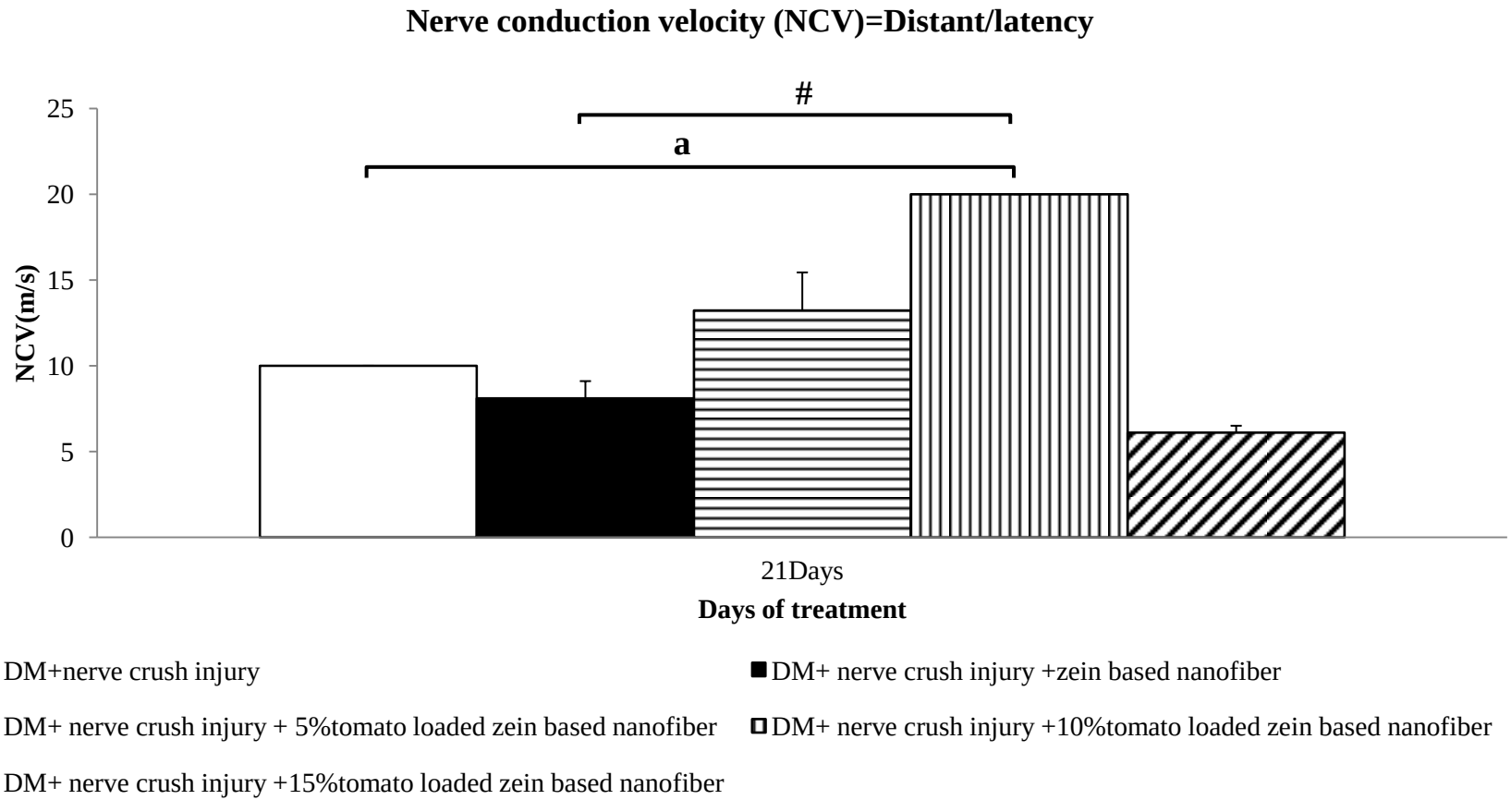
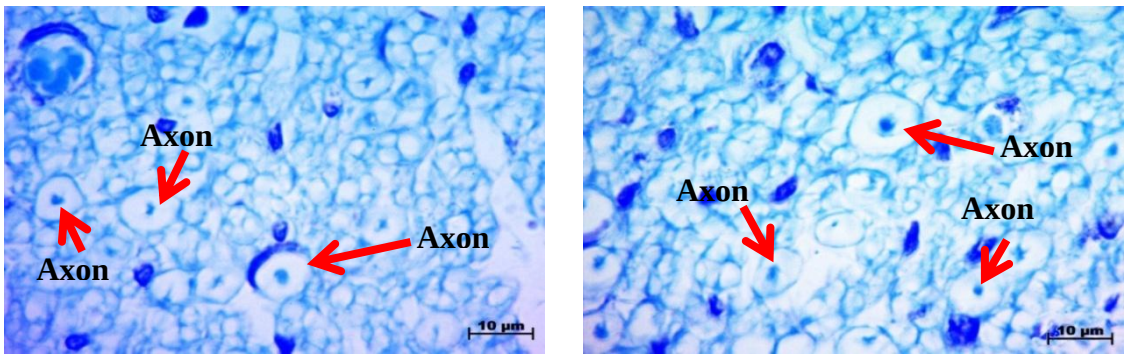


Figure 4-9 Effect of tomato extract loaded zein based nanofiber mat on nerve conduction velocity (NCV) of diabetic rats with crush injury (n=8/group). [a] P-value< .05; compared to DM+ nerve crush injury group. [#] P-value<.05; compared DM+ nerve crush injury +zein based nanofiber group

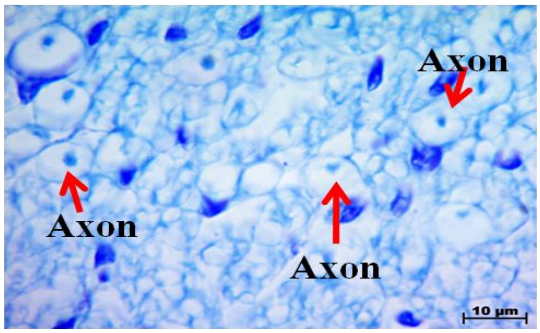
(A)

DM+ nerve crush injury

DM+ nerve crush injury +zein based nanofiber



DM+ nerve crush injury + 5%tomato loaded zein based nanofiber



DM+ nerve crush injury + 10%tomato loaded zein based nanofiber

DM+ nerve crush injury +15%tomato loaded zein based nanofiber

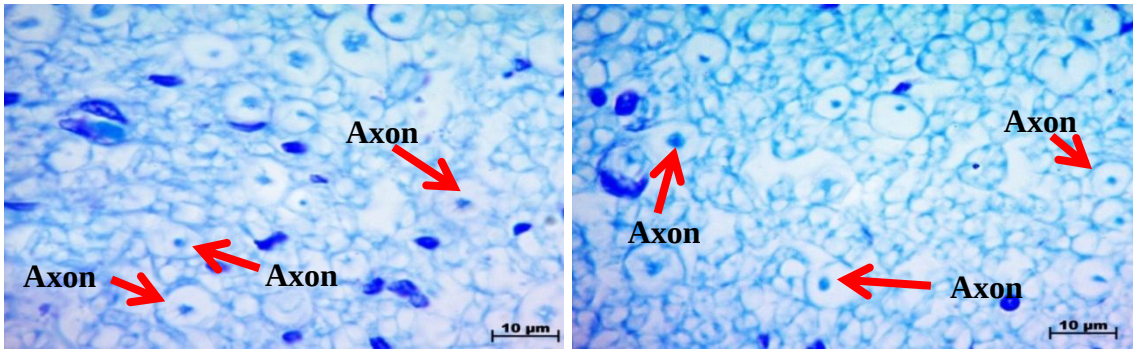


Figure 4-10 Effect of tomato extract loaded zein based nanofiber mat on axon density of sciatic nerve of diabetic rats with crush injury.

(A) Photographs of the lesion nerve stained with toluidine blue at 100X magnification.

(B) Bar graph showing the density of axon in the lesion nerve of various treatments groups. (n=8/group)

(B)

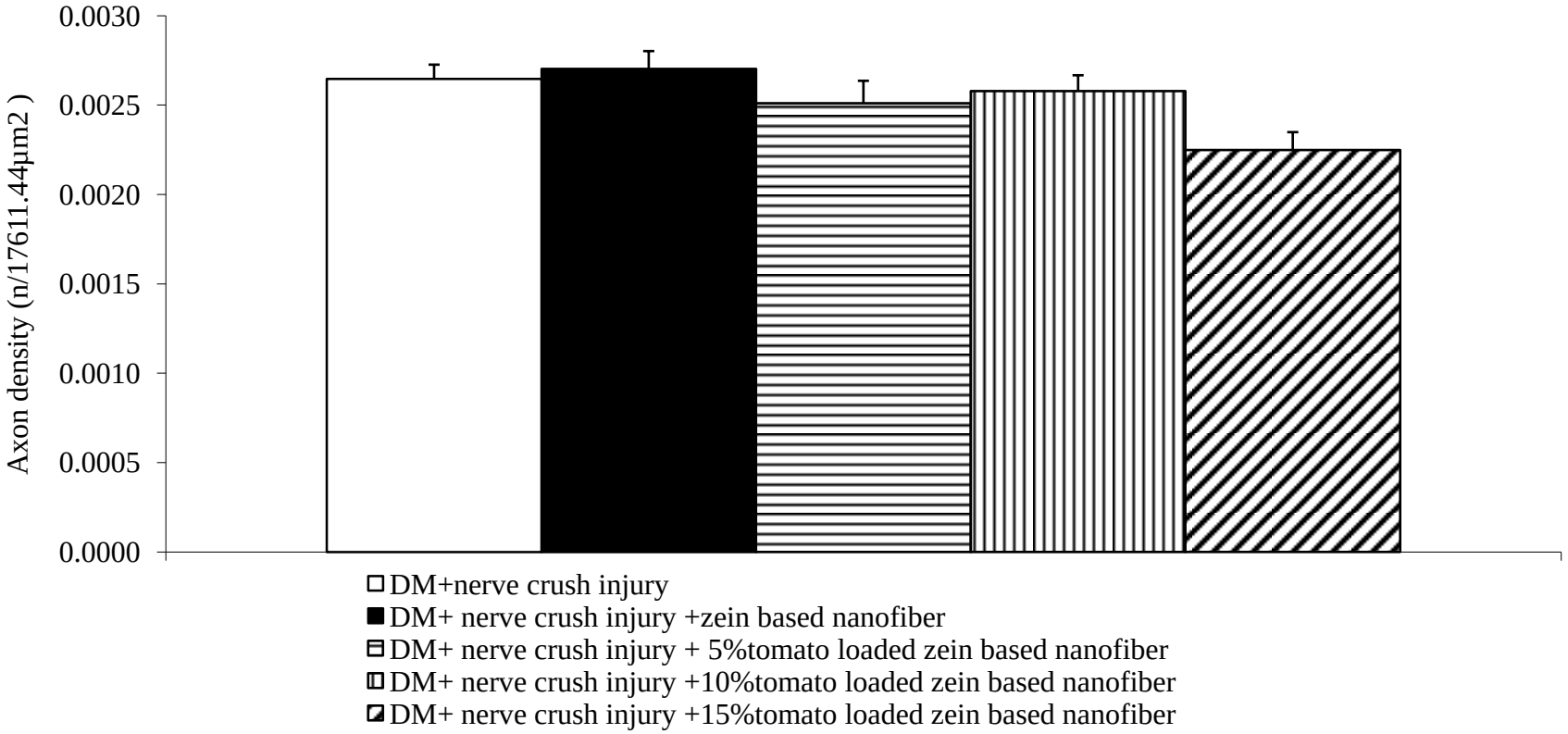


Figure 4-10 Effect of tomato extract loaded zein based nanofiber mat on axon density of sciatic nerve of diabetic rats with crush injury

(A) Photographs of the lesion nerve stained with toluidine blue at 100X magnification

(B) Bar graph showing the density of axon in the lesion nerve of various treatments groups. (n=8/group) (Cont.)

Table 4-11 Effect of tomato extract loaded zein based nanofiber mat on oxidative stress markers of sciatic nerve of diabetic rats with crush injury (n=8/group)

Group	MDA level (nmol/min.g.protein)	SOD activity (u/mg. protein)	CAT activity (u/mg. protein)	GPx.activity (u/mg. protein)
DM+nerve crush injury	$4x10^{-4}$±0.000	3.22±0.02	0.06±0.00	0.73±0.02
DM+ nerve crush injury +zein based nanofiber	$37x10^{-4}$±0.001[a]	3.29±0.10	0.59±0.21	0.75±0.06
DM+ nerve crush injury + 5%tomato loaded zein based nanofiber	$43x10^{-4}$±0.001[a]	9.81±0.50[a##]	0.41±0.16	1.87±0.09[aa##]
DM+ nerve crush injury +10%tomato loaded zein based nanofiber	$41x10^{-4}$±0.005[a]	3.60±0.50[#]	1.55±0.12[a#]	2.45±0.30[#]
DM+ nerve crush injury+15%tomato loaded zein based nanofiber	$53x10^{-4}$±0.001[a]	5.90±0.34[a#]	0.36±0.11	3.22±0.53[a#]

[a, aa, aaa] P-value< .05, .01 and .001 respectively; compared to DM+ nerve crush injury group.

[#, ##, ###] P-value<.05, .01 and .001 respectively; compared to DM+crush injury +zein based nanofiber group.

4. Effect of Quercetin Loaded Zein Based Nanofiber on the Functional Recovery of Sciatic Nerve of Diabetic Rats

It has been shown that oxidative stress also plays the crucial role on the nerve destruction after injury (Rotshenker, 2011; Senoglu et al., 2009). The current study had clearly demonstrated that zein based naofiber mat loaded with quercetin at concentrations of 5%, 10% and 15% decreased oxidative stress status in the lesion nerve of sciatic rats with crush injury together with the increased SOD, CAT and GPX enzymes in the lesion nerve. In addition, most of the functions of sciatic nerve which were associated with the large fiber such as the motor function showed the full recovery within 21 day-study period whereas the foot withdrawal reflex in response to heat stimuli which involved the unmyelinated fiber failed to show the full recovery at the end of study. Therefore, the functional recovery of sciatic nerve function induced by quercetin at all concentrations used in this study might occur partly via the improved oxidative stress status in the lesion nerve by enhancing the activities of scavenger enzymes mentioned earlier giving rise to the increased axon density in the lesion nerve.

ERK, a serine/threonine protein kinase, transduces extracellular stimuli into the responses of intracellular posttranslational and transcriptional processes which in turn promote the cell survival, growth, differentiation, and maintenance of phenotype (Rubinfield and Seger, 2005). Recent finding has demonstrated that the signal transduction of myelination is associated with the pERK1/2 (Fyffe-Maricich et al., 2013). In addition, ERK1/2 also contributes the essential role on the establishment of a regeneration-promoting extracellular environment for nerve to recover after injury (Sheu et al., 2000). The proliferation of Schwann cell, a cell type playing an important role on nerve regeneration and myelination, is also under the influence of ERK1/2. The stimulation of Schwann cell proliferation induced by growth factors requires the phosphorylation of ERK1/2. Therefore, the increased expression of pERK1/2 in diabetic rats with nerve crush injury which received zein based nanofiber mat loaded with 5% and 10% quercetin observed at 3 and 6 days respectively might play the role on the improved nerve regeneration and myelination which in turn improved the functional recovery of nerve function. However, quercetin might not exert the direct effect on the increased pERK1/2. It might exert the negative relationship with the mediator

which in turn increased pERK1/2 in the sciatic nerve. Therefore, the 10%quercetin loaded zein based nanofiber mat could release the suppression effect on the mediator which enhanced pERK1/2 late than 5%quercetin loaded zein based nanofiber mat. The increased pERK1/2 might also play the role on the increased axon density in the lesion nerve. Since this study didn't find the increased pERK1/2 of the high dose of quercetin (15%) and the time window of ERK1/2 and pERK1/2 and the alteration of axon density throughout a 21 day-experimental period, further researches are still essential to confirm the role of phosphorylation of ERK1/2 on the increased axon density.

The present study also demonstrated that the sensory and motor nerve fiber showed different vulnerability to quercetin. The thermosensation nerve fiber especially the unmyelinated nerve fiber was stimulated with high temperature via hot plate failed to show the full recovery within a 21 day- study period whereas the motor nerve fibers which are large myelinated nerve fibers showed the full recovery within the study period. This was in agreement with previous study which showed the different vulnerability between the unmyelinated and myelinated nerve fiber (Reeves et al., 2005; Hofmeijer et al., 2013).In addition, unmyelinated nerve fiber also exhibited slower recovery than myelinated fiber. The possible explanation was associated with the more rapid of the damage progression of the unmyelinated nerve fiber (Reeves et al., 2005). Since the unmyelinated nerve fiber had more structural damage, it required more time to recover when compared to myelinated nerve fiber. Therefore, the sensory function of sciatic nerve of diabetic rats with crush injury and received quercetin in this study failed to gain full recovery within the study period whereas the motor functions such as sciatic function index (SFI), walking pattern and motor power of the rats just mentioned showed the full recovery within the study period.

Zein based nanofiber mat loaded with quercetin is the novel potential candidate to improve the functional recovery of neuropathy in diabetic condition. Zein based nanofiber mat loaded with quercetin could enhance the functional recovery of nerve in diabetic condition. The motor functions which involve the large myelinated nerve fibers are more sensitive to quercetin loaded nanofiber mat. The possible underlying mechanism might occur via the improved oxidative stress status and the enhanced phosphorylation of ERK1/2 in the lesion nerve. These changes in

turn increased axon density and myelination leading to the improved nerve conduction velocity and the functional recovery of the lesion nerve in diabetic rats.

In conclusion, zein based nanofiber mat loaded with quercetin enhances the functional recovery in experimental diabetic neuropathy. Therefore, it may be served as the health product to facilitate the recovery of nerve. However, further researches are required to explore the effect of quercetin loaded nanofiber in the more severity model and to investigate the detail of underlying mechanism.

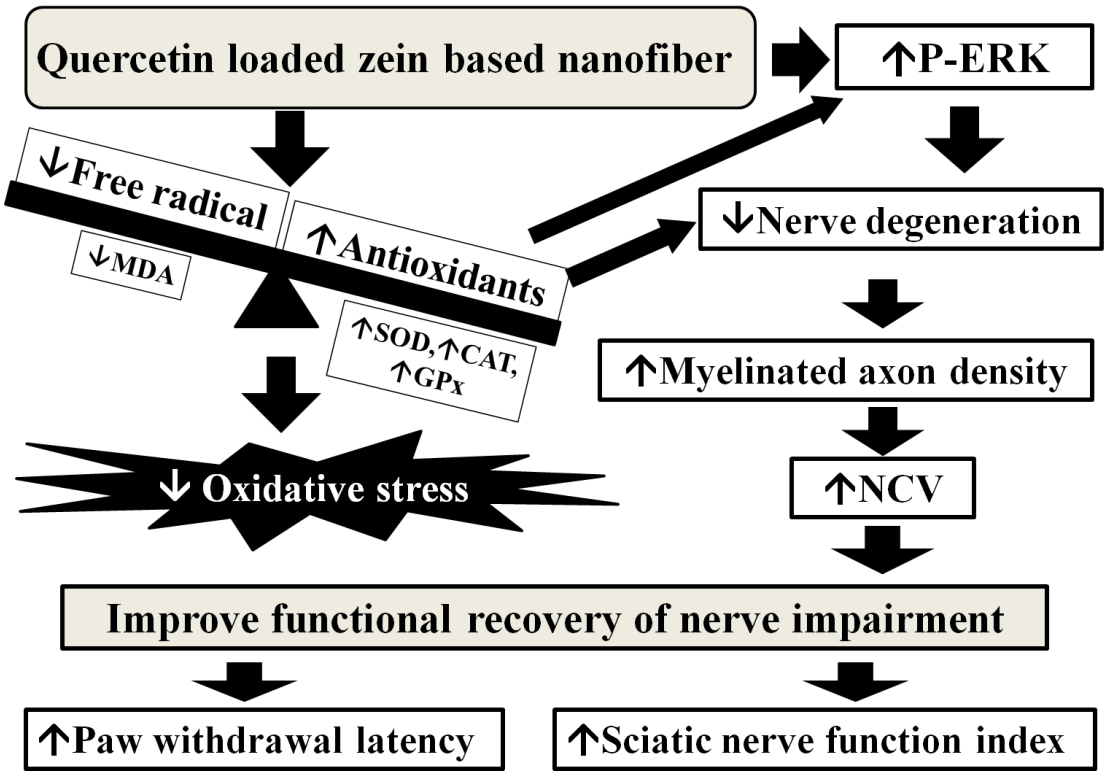


Figure 4-12 Schematic diagram shows the possible underlying mechanism of quercetin loaded zein based nanofiber on the functional recovery of sciatic nerve of diabetic rats

5. Effect of Tomato Extract Loaded Zein Based Nanofiber on the Functional Recovery of Sciatic Nerve of Diabetic Rats

The current study clearly demonstrated that zein based nanofiber mat loaded with tomato extract enhanced the functional recovery of nerve impairment in diabetic condition. It was found that only 5%tomato extract loaded zein based nanofiber mat could

enhance the sensory recovery of sciatic nerve after injury in diabetic rats while the motor recovery of sciatic nerve was observed in both diabetic rats which received 5% and 10% tomato extract loaded zein based nanofiber mat. This might occur because at low concentration of tomato extract could exert the beneficial effect on both large myelinated fiber and non-myelinated nerve fiber but at high concentration some ingredients in the extract might masking the effect of active ingredient on the non-myelinated nerve fiber. Therefore, no improvement of foot withdrawal reflex in response to heat stimuli was observed.

Since oxidative stress plays a pivotal role on the nerve destruction (Rotshenker, 2011; Senoglu et al., 2009) leading to nerve dysfunction, we also investigated the effect of tomato extract loaded zein based nanofiber mat on oxidative stress markers. Although tomato extract loaded zein based nanofiber mat increased antioxidant enzyme activity, no significant reduction of MDA level was observed. Therefore, oxidative stress might not play a role on the improved functional recovery of nerve impairment in diabetic rats. Based on the increased nerve conduction velocity (NCV) without the significant changes of nerve density, it was suggested that the improved NCV observed in diabetic rats which received 10% tomato extract loaded zein based nanofiber mat might be associated with the increased myelination or the increased intermodal length of myelin sheath. Since no change of oxidative stress was observed in this study, the underlying mechanism how tomato extract enhanced functional recovery might not relate with oxidative stress. On the basis of previous findings that phosphorylation of ERK1/2 play the crucial role on the establishment of a regeneration-promoting extracellular environment for nerve to recover after injury (Sheu et al., 2000) and myelination (Fyffe-Maricich et al., 2013), it was suggested that tomato extract loaded zein based nanofiber might enhance pERK1/2 in the lesion nerve which in turn increased both myelination and axon regeneration to the target tissue leading to the improved functional recovery especially the motor function of the lesion nerve. However, further researches are necessary to explore the effect of tomato extract loaded zein based nanofiber on signal transduction via ERK1/2.

Since tomato extract used in this study is the crude extract which contained numerous ingredients and the functional recovery of nerve in diabetic condition is complex, no simple relationship between the parameters playing a role on functional

recovery of nerve following injury and the concentrations of tomato extract wad observed and no dose dependent effect was presented.

In conclusion, this study is the first study to demonstrate the benefit of tomato extract loaded zein based nanofiber mat to enhance functional recovery of nerve impairment in diabetic condition. The possible underlying mechanism might not involve the improved oxidative stress status in the lesion nerve. The understanding about the precise underlying mechanism is still required further investigation.

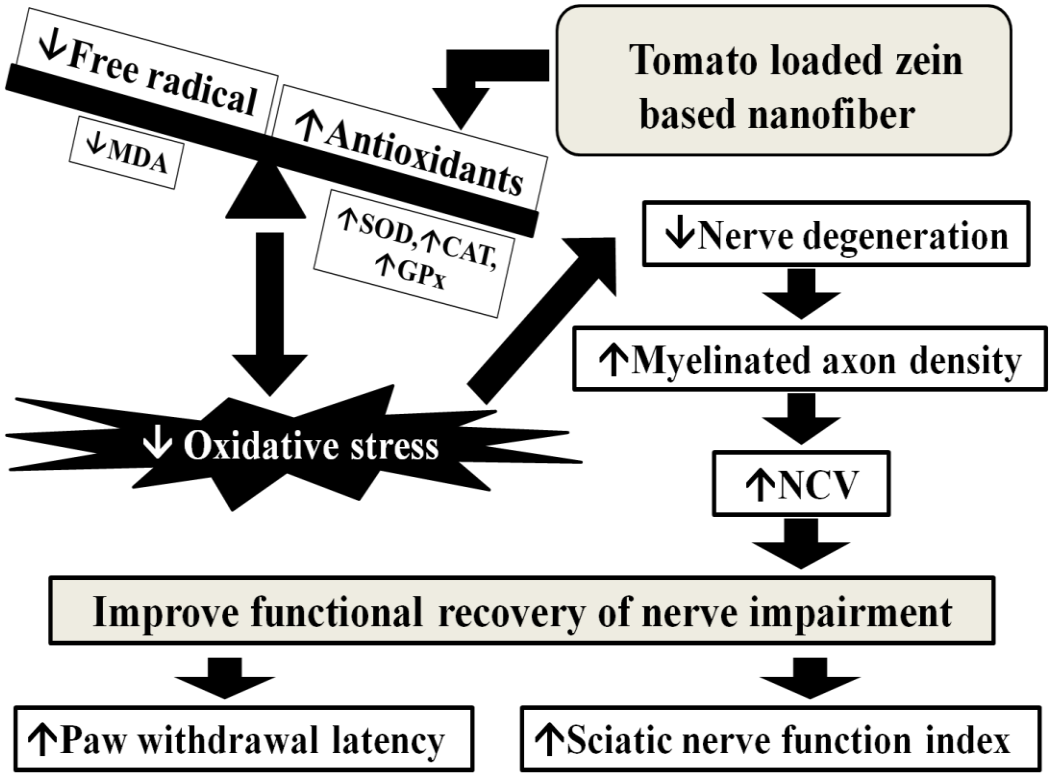


Figure 4-13 Schematic diagram shows the possible underlying mechanism of tomato extract loaded zein based nanofiber on the functional recovery of sciatic nerve of diabetic rats

6. Conclusion

Both quercetin and tomato extract loaded zein based nanofiber mats are the potential health products which may be benefit for diabetic neuropathy. However, the study in many models of diabetic neuropathy may be necessary to assure the beneficial effects of the novel innovations before moving to clinical trial.