Enriching the diet with menhaden oil improves peripheral neuropathy in streptozotocin-induced type 1 diabetic rats

Lawrence J. Coppey², Eric P. Davidson², Alexander Obrosov² and Mark A. Yorek¹,²,³,⁴.

¹Department of Veterans Affairs Iowa City Health Care System, Iowa City, IA, 52246
²Department of Internal Medicine, University of Iowa, Iowa City, IA, 52242
³Veterans Affairs Center for the Prevention and Treatment of Visual Loss, Iowa City, IA, 52246
⁴Fraternal Order of Eagles Diabetes Research Center, University of Iowa, Iowa City, IA, 52242

Contribution statement: LJC, EPD, AO and MAY performed experiments; MAY designed the experiments and wrote the manuscript

Running title: n-3 fatty acid enrichment and neuropathy in type 1 diabetes

Correspondence should be addressed to:
Mark A. Yorek
Room 204, Building 40
Department of Veterans Affairs Iowa City Health Care System, Iowa City, IA 52246
Tel #: 1-319-338-0581 ext. 7696
Fax # 1-319-339-7162
E-mail: mark-yorek@uiowa.edu

Copyright © 2014 by the American Physiological Society.
The purpose of this study was to determine the effect of supplementing the diet of type 1 diabetic rats with menhaden oil on diabetic neuropathy. Menhaden oil is a natural source for n-3 fatty acids, which have been shown to have beneficial effects in cardiovascular disease and other morbidities. Streptozotocin-induced diabetic rats were used to examine the influence of supplementing their diet with 25% menhaden oil on diabetic neuropathy. Both prevention and intervention protocols were used. Endpoints included motor and sensory nerve conduction velocity, thermal and mechanical sensitivity and innervation and sensitivity of the cornea and hindpaw. Diabetic neuropathy as evaluated by the stated endpoints was found to be progressive. Menhaden oil did not improve elevated HbA1C levels or serum lipid levels. Diabetic rats 16 weeks duration were thermal hypoalgesic, had reduced motor and sensory nerve conduction velocities and innervation and sensitivity of the cornea and skin were impaired. These endpoints were significantly improved with menhaden oil treatment following the prevention or intervention protocol. We found that supplementing the diet of type 1 diabetic rats with menhaden oil improved a variety of endpoints associated with diabetic neuropathy. These results suggest that enriching the diet with n-3 fatty acids may be a good treatment strategy for diabetic neuropathy.

Key words: diabetic peripheral neuropathy, fish oil, epidermal nerve fibers, corneal nerve fibers, type 1 diabetes
INTRODUCTION

It is generally accepted that increased consumption of n-3 fatty acids lowers the risk of cardiovascular disease (De Caterina 2011). The main source of n-3 fatty acids in the Western diet is fish, especially oily fish (De Caterina 2011). Over several decades a large number of studies have found an inverse association between fish consumption and morbidity and mortality from coronary heart disease (Calder 2004; De Caterina 2011; Kris-Etherton et al. 2002). Blood levels of n-3 fatty acids also appear to correlate inversely with death from cardiovascular causes (Albert et al. 2002; De Caterina 2011; Siscovick et al. 1995). Less is known about the effects of n-3 fatty acids on diabetic complications such as neuropathy.

Peripheral neuropathy affects over 50% of patients with diabetes and is responsible for the majority of non-trauma related amputations. To date, tight glycemic control is the only strategy shown to prevent or delay the development of neuropathy in patients with type 1 diabetes (Ang et al. 2014). However, the DCCT study showed that tight glycemic control is difficult to achieve and sustain over time and, perhaps insufficient to fully prevent diabetic neuropathy (Ang et al. 2014). Thus, there is a significant unmet need for an effective and safe treatment for diabetic neuropathy. Our group has found that fish oil, a natural source of n-3 fatty acids, supplementation of type 2 diabetic rats improved diabetic neuropathy (Ang et al. 2014). In patients with type 2 diabetes long-term treatments with eicosapentanoic acid, an n-3 fatty acid had beneficial effects on diabetic neuropathy (Okuda et al. 1996). To further explore the benefits of dietary n-3 fatty acid enrichment on diabetic neuropathy we performed a pre-clinical study using type 1 diabetic rats and both a prevention and intervention protocol.
MATERIALS AND METHODS

Materials: Unless stated otherwise all chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals: Male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats 10-11 weeks of age were housed in a certified animal care facility and food (Harlan Teklad, #7001, Madison, WI) and water were provided ad libitum. All institutional (approval ACURF #1290701) and NIH guidelines for use of animals were followed. As a preliminary study we examined the progression of diabetes-induced neuropathic changes over a period of 12 weeks. For this study rats at 12 weeks of age were separated into two groups. One of these groups was treated with streptozotocin (55 mg/kg in 0.1M citric acid buffer, pH 4.5, i.p.). Diabetes was verified 96 h later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Aviva Accu-Chek, Roche, Mannheim, Germany). Rats having blood glucose level of 300 mg/dl (11.1 mM) or greater were considered to be diabetic. The other group was treated with vehicle and was termed the control group. All diabetic rats were treated with 2-3 units of Lantus insulin every other day in order to maintain body weight (Oltman et al. 2011). At 4, 8 and 12 weeks rats from both the control and diabetic groups were examined for neuropathic endpoints.

For the study to examine the effect of enrichment of the diet with menhaden oil on diabetic neuropathy rats at 12 weeks of age were separated into five groups. Three of these groups were treated with streptozotocin and diabetes verified as described above. One group of diabetic rats (diabetic non-treated) remained on the standard diet for the entire 16 weeks of the study. A second group of diabetic rats (prevention group) was placed on a diet containing 25% kcal fat derived from menhaden oil immediately after verification of hyperglycemia (Research Diets, New Brunswick, NJ). The third group of diabetic rats (intervention group) remained on the standard diet for 8 weeks and then was placed on the menhaden oil enriched diet for the final 8 weeks of the study. The other two groups of rats not treated with streptozotocin were fed
a standard diet (control group) or placed on the menhaden oil enriched diet (control treated) for the 16 week period of the study. The fatty acid composition of the standard diet and the diet enriched with menhaden are provided in Table 1.

**Behavioral response:** Thermal nociceptive response in the hindpaw was measured using the Hargreaves method as previously described (Oltman et al. 2008). Briefly, the rat was placed in the observation chamber on top of the thermal testing apparatus and allowed to acclimate to the warmed glass surface (30°C) and surroundings for a period of 15 min. The mobile heat source was maneuvered so that it was under the heal of the hindpaw and then activated, a process that activates a timer and locally warms the glass surface, when the rat withdrew its paw, the timer, and the heat source was turned off and the time was recorded. The timer was defaulted to go off after 25 sec to avoid injury to the rat. Following an initial recording, which was discarded, two measurements were made for each hindpaw, with a rest period of 5 min between each measurement. The mean of the measurements reported in sec were used as the thermal nociceptive response. Tactile responses were evaluated by quantifying the withdrawal threshold of the hindpaw in response to stimulation with flexible von Frey filaments as previously described (Drel et al. 2007). The data was reported in grams. Corneal sensation was measured using a Cochet-Bonnet filament esthesiometer (Luneau Ophtalmogie, France) as previously described (Davidson et al. 2012). The testing began with the nylon filament extended to the maximal length (6 cm). The end of the nylon filament was touched to the cornea. If the rat blinked (positive response) the length of the filament was recorded. If the rat did not blink then the nylon filament was shortened by 0.5 cm and the test repeated until a positive response was recorded. This process was repeated for each eye three times. The data was reported as cm.

**Motor and sensory nerve conduction velocity:** On the day of terminal studies rats were weighed and anesthetized with sodium pentobarbital, i.p. (50 mg/kg, Abbott Laboratories, North Chicago, IL). Motor nerve conduction velocity was determined as previously described using a noninvasive procedure in the sciatic-posterior tibial conducting system (Coppey et al. 2000).
The left sciatic nerve was stimulated first at the sciatic notch and then at the Achilles tendon. Stimulation consisted of single 0.2-ms supramaximal (8 V) pulses through a bipolar electrode (Grass S44 Stimulator, Grass Medical Instruments, Quincy, MA). The evoked potentials were recorded from the interosseous muscle with a unipolar platinum electrode and displayed on a digital storage oscilloscope (model 54600A, Hewlett Packard, Rolling Meadows, IL). Motor nerve conduction velocity was calculated by subtracting the distal from the proximal latency measured in milliseconds from the stimulus artifact of the take-off of the evoked potential and the difference was divided into the distance between the 2 stimulating electrodes measured in millimeters using a Vernier caliper. Sensory nerve conduction velocity was determined using the digital nerve as described by Obrosova et al. (2004). Briefly, hind limb sensory nerve conduction velocity was recorded in the digital nerve to the second toe by stimulating with a square-wave pulse of 0.05-ms duration using the smallest intensity current that resulted in a maximal amplitude response. The sensory nerve action potential was recorded behind the medial malleolus. The sensory nerve conduction velocity was calculated by measuring the latency to the onset/peak of the initial negative deflection and the distance between stimulating and recording electrodes. The motor and sensory nerve conduction velocity was reported in meters per second.

Corneal innervation: Subbasal corneal nerves were imaged using the Rostock cornea module of the Heidelberg Retina Tomograph confocal microscope as previously described (Davidson et al. 2012). The anesthetized rat was secured to a platform that allows adjustment and positioning of the rat in three dimensions. A drop of GenTeal (lubricant eye gel) was applied onto the tip of the lens and advanced slowly forward until the gel contacted the cornea allowing optical but not physical contact between the objective lens and corneal epithelium (Davidson et al. 2012). Six random high-quality images without overlap of the sub-epithelial nerve plexus around the perimeter of the central cornea were acquired by finely focusing the objective lens to maximally resolve the nerve layer just under the corneal epithelium. The
investigator acquiring these images was masked with respect to identity of the animal condition.

For these studies a single parameter of corneal innervation was quantified. Corneal nerve fiber length defined as the total length of all nerve fibers and branches (in millimeters) present in the acquired image standardized for area of the image (in square millimeters) was determined for each image by tracing the length of each nerve in the image, summing the total length and dividing by the image area (Davidson et al. 2012). The corneal fiber length for each animal was the mean value obtained from the six acquired images and expressed as mm/mm². Based on receiver operating characteristic (ROC) curve analysis corneal nerve fiber length is the optimal parameter for diagnosing patients with diabetic neuropathy and has the lowest coefficient of variation (Quattrini et al. 2007; Tavakoli et al. 2010).

After completion of all in vivo analyses corneas were dissected from the eyes and trimmed around the scleral-limbal region as previously described (Yorek et al. 2014). The cornea was fixed for 1h in 2% paraformaldehyde in phosphate-buffered saline. The tissue was blocked with 0.2% Triton X-100 and 1% bovine serum albumin (BSA) for 1h, and then permeabilized in 10% Triton X-100 and 1% bovine serum albumin for 1h. The cornea was then incubated with neuronal class III β-anti-tubulin 1:500 in incubation buffer overnight at 4°C (Covance, Dedham, MA). After washing with incubation buffer, the tissue was incubated with Alexa Fluor 546 goat anti-rabbit IgG 1:2000 in incubation buffer for 2 hours at room temperature (Invitrogen, Eugene, OR). After washing, the cornea was placed epithelium up on a microscope slide. Excess water was carefully aspirated and three radial cuts were made at 120 degree intervals, nearly to the center of the cornea. The tissue was carefully covered with a cover slip, mounted with ProLong Gold and sealed with clear nail polish. A 3x3 matrix of Z-stack images were collected using a Zeiss LM710 confocal microscope with ZEN Black software. An analysis of corneal nerve images was completed with Imaris software version 7.6.4 X64 (Bitplane, Zurich, Switzerland). For epithelial corneal nerves to determine the total surface area covered by corneal innervation
a two-dimensional surface was rendered on the fluorescent staining of a maximum projection image. The measurements are reported as a percentage of total area.

**Intraepidermal nerve fiber density in the hindpaw:** Immunoreactive intraepidermal nerve fiber profiles, which are primarily sensory nerves, were visualized using confocal microscopy. Samples of skin of the right hindpaw were fixed, dehydrated and embedded in paraffin. Sections (7 µm) were collected and immuno stained with anti-PGP9.5 antibody (rabbit anti human, AbD Serotic, Morpho Sys US Inc., Raleigh, NC) overnight followed by treatment with secondary antibody Alexa Fluor 546 goat anti rabbit (Invitrogen, Eugene, OR). Profiles were counted by two individual investigators that were masked to the sample identity. All immunoreactive profiles within the epidermis were counted and normalized to epidermal length (Davidson et al. 2010, 2012).

**Biological and oxidative stress markers:** Non-fasting blood glucose was determined. Hemoglobin A1C levels were determined using a Glyco-tek affinity column kit (Helena Laboratories, Beaumont, TX). Serum samples were collected for determination of free fatty acid, triglyceride and free cholesterol using commercial kits from Roche Diagnostics, Mannheim, Germany; Sigma Chemical Co., St. Louis, MO; and Bio Vision, Mountain View, CA, respectively. Serum samples were also collected for analysis of fatty acid composition. Lipids were extracted from diets and serum with a 2:1 (vol/vol) mixture of chloroform and methanol followed by phase separation with a solution of 154 mM NaCl and 4 mM HCl. Fatty acid composition were measured after the lipid fraction was transesterified in 14% boron trifluoride in methanol and the fatty acid methyl esters extracted into heptane before separation by gas-liquid chromatography (Yorek et al. 1984a, 1984b). Individual fatty acids peaks as % of total fatty acids present were identified by comparison to known fatty acid standards.

**Data Analysis:** Results are presented as mean ± S.E.M. Comparisons between control and non-treated diabetic rats were conducted using Student t-test (Prism software; GraphPad, San Diego, CA). Comparison between control, non-treated and treated diabetic rats were conducted
using one-way ANOVA and Bonferroni posttest comparison (Prism software; GraphPad, San Diego, CA). A P value of less than 0.05 was considered significant.
RESULTS

Effect of type 1 diabetes duration 4-12 weeks on neuropathy. Table 2 presents data for the progression of diabetic neuropathy over the period of 4-12 weeks after the induction of hyperglycemia in 12 week old rats. From 4 to 12 weeks hemoglobin A1C levels trended to increase. Both motor and sensory nerve conduction velocities were significantly decreased compared to control rats after 4 weeks of diabetes. Diabetic rats were thermal hypoalgesic after 8 weeks of hyperglycemia. In this study after 4 weeks of diabetes we did not observe any indication of thermal hyperalgesia. In the cornea a significant decrease of sub-epithelial corneal nerves was detected using corneal confocal microscopy after 8 weeks of diabetes and a significant decrease in corneal sensitivity was detected using a Cochet-Bonnet filament esthesiometer after 12 weeks of diabetes. Based on these results we chose the time frame for the intervention protocol to be 8 weeks of non-treated diabetes followed by 8 weeks of treatment.

Effect of type 1 diabetes and dietary treatment with menhaden oil on serum fatty acid composition. Data in Table 3 show the fatty acid composition of the serum of control rats treated with or without menhaden oil, non-treated diabetic rats and diabetic rats treated with menhaden oil following a prevention or intervention protocol. Compared to serum from control rats there is little change in the fatty acid composition in the serum from diabetic rats. Treating control rats for 16 weeks with menhaden oil caused a significant decrease in oleic acid, linoleic acid and arachidonic acid in the serum and a significant increase in eicosapentaenoic acid and docosahexaenoic acid compared to control rats. Treating diabetic rats with menhaden oil caused a significant decrease in stearic acid, linoleic acid and arachidonic acid in the serum and a significant increase in eicosapentaenoic acid and docosahexaenoic acid compared to control or non-treated diabetic rats. There was no difference in the fatty acid composition of serum in diabetic rats treated with menhaden oil for 8 (intervention) or 16 (prevention) weeks. As
expected the fatty acid unsaturation index was significantly increased in serum from control or
diabetic rats treated with menhaden oil compared to control or non-treated diabetic rats (Table
3). The n-6 to n-3 fatty acid ratios in serum from control, control + menhaden oil, non-treated
diabetic, diabetic + menhaden oil (prevention) and diabetic + menhaden oil (intervention) rats
was 16.3 ± 1.2, 1.5 ± 0.1, 14.2 ± 1.9, 1.5 ± 0.1 and 1.8 ± 0.1, respectively.

Effect of type 1 diabetes and dietary treatment with menhaden oil on weight, blood
glucose and serum lipid levels. Data in Table 4 demonstrate that non-treated and treated
diabetic rats failed to gain weight compared to control rats. Control rats fed the menhaden oil
enriched diet trended to gain more weight than control rats but the difference in the final weight
was not significant. Blood glucose and hemoglobin A1C values were significantly increased in
non-treated diabetic rats and treating diabetic rats with menhaden oil did not significantly affect
the hyperglycemic state. Serum triglycerides, free fatty acids and cholesterol were all
significantly increased in non-treated and treated diabetic rats. Treating control rats with
menhaden oil did not affect blood glucose or serum lipid levels.

Effect of type 1 diabetes and dietary treatment with menhaden oil on nerve conduction
velocity, thermal nociception, tactile response and intraepidermal nerve fiber density. Motor and
sensory nerve conduction velocity was significantly decreased non-treated diabetic rats
compared to control rats (Figure 1). Treating diabetic rats using a prevention or intervention
protocol with a high fat diet enriched with menhaden oil significantly improved motor and
sensory nerve conduction velocity compared to non-treated diabetic rats although motor nerve
conduction velocity in diabetic rats treated with menhaden oil remained significantly decreased
compared to control rats. Treating control rats with menhaden oil did not affect motor or
sensory nerve conduction velocity. Data in Figure 2 demonstrate that non-treated diabetic rats
are hypoalgesic to thermal stimuli compared to control rats and this was significantly improved
when diabetic rats were treated using either a prevention or intervention protocol with a high fat
diet enriched with menhaden oil. Tactile response threshold was significantly decreased in non-
treated diabetic rats (Figure 2). Treating diabetic rats with a diet enriched with menhaden oil using a prevention protocol significantly improved the tactile response. The tactile response in diabetic rats treated with dietary menhaden oil using an intervention protocol was also improved but to a lesser extent than observed using the prevention protocol. Treating control rats with a diet enriched with menhaden oil did not affect thermal or tactile responses (Figure 2).

Intraepidermal nerve fiber profiles in the hindpaw of non-treated diabetic rats were significantly decreased compared to control rats (Figure 3). Treating diabetic rats with a diet enriched with menhaden oil significantly improved intraepidermal nerve fiber density. Treating diabetic rats using a prevention protocol significantly increased intraepidermal nerve fiber profiles compared to control rats. Treating control rats with a diet enriched with menhaden oil for 16 weeks also significantly increased intraepidermal nerve fiber profiles compared to control rats.

Effect of type 1 diabetes and dietary treatment with menhaden oil on epithelial and sub-epithelial corneal nerve fibers and cornea sensitivity. Data in Figures 3 and 4 demonstrate that corneal nerves of the sub-epithelial layer (Figure 4) and epithelium (Figure 3) are significantly decreased in diabetic rats. Treating diabetic rats with a diet enriched with menhaden oil prevented and/or reversed the loss in corneal nerves. Treating diabetic rats with menhaden oil also prevented the diabetes-induced decrease in cornea sensitivity (Figure 4). Treating control rats with menhaden oil had no significant effect on corneal nerve density in the sub-epithelial layer or epithelium or on cornea sensitivity. Figure 5 provides representative images of intraepidermal nerve fibers in the skin from a hindpaw (A), sub-epithelial corneal nerves obtained using corneal confocal microscopy (B), sub-epithelial corneal nerves in the region of the whorl obtained following immunohistochemical staining of the nerves with β-anti-tubulin and visualization using standard confocal microscopy (C) and corneal nerves of the epithelium in the region of the whorl obtained following immunohistochemical staining of the nerves with β-anti-tubulin and visualization using standard confocal microscopy (D). All images were obtained from a control animal.
DISCUSSION

The goal of these studies was to determine whether enriching the diet of type 1 diabetic rats with menhaden oil, a natural source of n-3 fatty acids, improves diabetic neuropathic endpoints. Fish oils are a common dietary supplement used for a variety of conditions including cardiovascular health and could be easily translated to clinical trials for diabetic peripheral neuropathy. Some of the unique features of this study were that we used 16 week duration for diabetes and incorporated both a prevention and intervention protocol into the study design with initiation of the intervention protocol beginning after neuropathy had developed. The hypothesis being tested was that treatment of diabetic rats with a source of n-3 fatty acids will promote a decrease in the n-6/n-3 fatty acid ratio, a sign of reduced inflammatory stress, leading to prevention and repair of diabetic neuropathy related endpoints. The endpoints examined included determination of the motor and sensory nerve conduction velocity as well as examination of nerve structure and functional changes in the skin and cornea. The determination of nerve conduction velocities are standard endpoints for the study of diabetic neuropathy, whereas examination of changes of structure and function of the small sensory nerve fibers in the skin or cornea have recently been promoted as markers of diabetic peripheral neuropathy and may provide a means for early detection (Loseth et al. 2008; Narayanaswamy et al. 2012; Pittenger et al. 2005; Quattrini et al. 2007).

The major findings from this study were that the development of diabetic neuropathology endpoints examined appeared at different times over the duration of 4-12 weeks of non-treated diabetes. Reduction in motor and sensory nerve conduction velocity was the first deficit to appear after 4 weeks of diabetes. Impairment of functional and structural deficits in the skin and cornea occurred after 8-12 weeks of diabetes. Treating diabetic rats with a diet enriched with menhaden oil from the onset of hyperglycemia prevented the development of the neuropathology observed in non-treated diabetic rats. More importantly, intervention after 8
weeks of non-treated diabetes with menhaden oil enriched diet reversed the neuropathological changes after only 8 weeks of treatment.

The serum fatty acid profile was not significantly different between control and diabetic rats. However, after 8-16 weeks of treatment with menhaden oil both control and diabetic rats had a significantly different serum fatty acid profile compared to untreated rats and reflects a new steady state with higher levels of the n-3 fatty acids eicosapentaenoic and docosahexaenoic acids. With higher levels of n-3 fatty acids accounting for a greater percentage of the fatty acids in serum there was a significant decrease in oleic, linoleic and arachidonic acids in control rats treated with menhaden oil compared to untreated control rats and a significant decrease in stearic, linoleic and arachidonic acids in diabetic rats treated with menhaden oil compared to untreated diabetic rats. Notable differences between control and diabetic rats treated with menhaden oil were that levels of oleic acid in serum of diabetic rats were significantly higher in diabetic rats compared to control rats treated with menhaden oil and levels of arachidonic acid in control rats treated with menhaden oil were significantly higher compared to diabetic rats treated with menhaden oil. The reason for these differences are not entirely clear but it appears that treating diabetic rats with menhaden oil tended to maintain levels of oleic acid in the serum at the expense of arachidonic acid. These changes in the serum fatty acid composition with menhaden oil treatment of diabetic rats resulted in a significant lowering of the n-6 to n-3 fatty acid ratio. Lowering of the n-6 to n-3 fatty acid ratio is a marker for reduction in inflammatory stress and could partially explain some of the beneficial effects of enriching the diet with menhaden oil on diabetic neuropathy. This suggests that the potential for inflammatory mediators being produced are significantly reduced in diabetic rats fed the menhaden oil enriched diet (Valenzuela and Videla 2011). n-3 Fatty acid enrichment is well known to have anti-inflammatory effects including increase in adiponectin production, an anti-inflammatory adipokine, and suppressing the activation of Toll-like receptor-4 (Kalupahana et al. 2011; Liu et al. 2013; Moreno-Aliaga et al. 2010; Siriwardhana et al. 2012; Tishinsky et al.
Increased inflammatory stress has long been considered a contributing factor to the development and progression of diabetic neuropathy and a target for therapeutic intervention (Cameron and Cotter 2008; Sytze Van Dam et al. 2013; Vincent et al. 2011). However, results from this study should not be interpreted as n-6 fatty acids being a risk factor for diabetic neuropathy. Several studies have demonstrated that treating diabetic rats with evening primrose oil, a source of γ-linolenic acid (an n-6 fatty acid), improves diabetic neuropathy as demonstrated by correction of impaired nerve conduction velocity, nerve blood flow and neurovascular function (Cameron and Cotter 1997; Dines et al. 1995; Head et al. 2000; Omran 2012; Tomlinson et al. 1989). Omran et al. (2012) demonstrated that diabetic rats treated with evening primrose oil showed fewer morphologic alterations with a decrease in myelin breakdown. It is thought that treating diabetic rats with evening primrose oil provides a source for γ-linolenic acid, which is decreased by diabetes thereby, improving the synthesis of eicosanoids important for vasodilation especially prostacyclin (Cameron and Cotter 1997; Omran 2012). Vascular dysfunction is thought to be a contributing factor to diabetic neuropathy and we have previously demonstrated that impaired vascular relaxation to acetylcholine by epineurial arterioles, blood vessels that supply the sciatic nerve, precedes slowing of nerve conduction velocity (Coppey et al. 2000). In this study we did not determine the effect of menhaden oil on vascular function or blood flow, a goal for future studies. However, we previously demonstrated that treating diet induced obese mice with menhaden oil corrected vasodilation to acetylcholine by arteries of the gracilis muscle (Lamping et al. 2013). Metabolites of eicosapentaenoic acid and docosahexaenoic acid, referred to as resolvins (resolution-phase interaction products) and neuroprotectin D1, have anti-oxidant, anti-inflammatory and neuro-protection properties (Ariel and Serhan 2007; Kohli and Levy 2009). Resolvins are oxygenated metabolites of eicosapentaenoic acid (E series resolvins) and docosahexaenoic acid (D series resolvins). In non-vascular tissue 15-lipoxygenase-1 is responsible for the generation of resolvins and neuroprotectin D1 and eicosapentaenoic acid.
and docosahexaenoic acid are good substrates for 15-lipoxygenase-1. Resolvin formation can be increased by consuming increased amounts of eicosapentaenoic acid or docosahexaenoic acid (Ariel and Serhan 2007; Kohli and Levy 2009). Regeneration of corneal nerves damaged by refractive surgery can be increased with treatment of docosahexaenoic acid through synthesis of neuroprotectin D1 (Cortina et al. 2010; Gordon and Bazan 2013). This group also reported that neuroprotectin D1 increases neurite outgrowth by trigeminal ganglia from Swiss Webster mice (Cortina et al. 2013). Robson, et al. (2010) reported that n-3 fatty acids promote neurite outgrowth by dorsal root ganglia and the effect of docosahexaenoic acid was still prominent in aged tissue. In addition to being a substrate for formation of bioactive metabolites and having anti-inflammatory properties, n-3 fatty acids have been shown to affect a range of molecular pathways including alteration of physical properties of cellular membranes, modulation of membrane channels and proteins and regulation of gene expression via nuclear receptors and transcription factors (Mozaffarian and Wu 2011). Membrane alteration with n-3 fatty acids has been shown to affect Akt signaling, impacting neuronal survival (Akbar et al. 2005).

In the current study we found that enrichment of the diet with menhaden oil of a rat model of type 1 diabetes improved endpoints associated with diabetic neuropathy. Our study did not address the mechanism(s) that may be responsible for the beneficial effects of menhaden oil on diabetic neuropathy. However, it is possible that enriching the diet of diabetic rats with menhaden oil contributed to an increase in resolvin and neuroprotectin D1 production and neural protection/regeneration. Future studies will examine the effect menhaden oil supplementation has on reducing inflammatory stress and promoting the formation of neuroprotective compounds such as resolvins and neuroprotectin D1.

In summary, we have demonstrated that dietary enrichment with menhaden oil, a natural source of n-3 fatty acids, in a rat model for type 1 diabetes prevented, but more importantly reversed, numerous pathological endpoints associated with diabetic neuropathy. These results are in
agreement with previous studies performed with a type1 and 2 diabetic rat models (Coppey et al. 2012; Gerbi et al. 1999). This suggests that dietary enrichment with n-3 fatty acids may be beneficial treatment for diabetic peripheral neuropathy.
This material is based upon work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development (BX001680-01), Rehabilitation Research and Development (RX000889-01), Iowa City VA Center of Excellence for the Prevention and Treatment of Visual Loss: C9251-C) and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK081147 from NIH. The content of this manuscript are new and solely the responsibility of the authors and do not necessarily represent the official views of the granting agencies.
The authors have no conflicts of interest, financial or otherwise to declare.
REFERENCES


Valenzuela R, Videla LA. The importance of the long-chain polyunsaturated fatty acid n-6/n-3 ration in development of non-alcoholic fatty liver associated with obesity. *Food Funct* 2: 644-648, 2011.


FIGURE LEGENDS

Figure 1: Effect of treatment of type 1 diabetic rats with menhaden oil (MO) supplemented diet using a prevention or intervention protocol on motor and sensory nerve conduction velocity. Motor and sensory nerve conduction velocity was determined as described in the Materials and Methods section. Data are presented as the mean ± S.E.M. in m/sec. The number of rats in each group was the same as shown in Table 3. * p < 0.05 compared to control rats; + p < 0.05 compared to non-treated diabetic rats.

Figure 2: Effect of treatment of type 1 diabetic rats with menhaden oil (MO) supplemented diet using a prevention or intervention protocol on thermal nociception and tactile response. Thermal sensitivity and tactile response threshold was determined as described in the Materials and Methods section. Data are presented as the mean ± S.E.M. in seconds for thermal sensitivity and grams for tactile response threshold. The number of rats in each group was the same as shown in Table 3. * p < 0.05 compared to control rats; + p < 0.05 compared to non-treated diabetic rats.

Figure 3: Effect of treatment of type 1 diabetic rats with menhaden oil (MO) supplemented diet using a prevention or intervention protocol on intraepidermal nerve fiber and epithelial corneal nerve density. Intraepidermal and epithelial corneal nerve fiber density was determined as described in the Materials and Methods section. Data are presented as the mean ± S.E.M. in profiles/mm² for intraepidermal nerve fiber density and area % for epithelial corneal nerve fiber density. The number of rats in each group was the same as shown in Table 2. * p < 0.05 compared to control rats; + p < 0.05 compared to non-treated diabetic rats.
Figure 4: Effect of treatment of type 1 diabetic rats with menhaden oil (MO) supplemented diet on sub-epithelial cornea nerve fiber length and cornea sensitivity. Sub-epithelial cornea nerve fiber length and cornea sensitivity was determined as described in the Materials and Methods section. Data are presented as the mean ± S.E.M. in mm/mm² for sub-epithelial nerve fiber length and cm for cornea sensitivity. The number of rats in each group was the same as shown in Table 2. * p < 0.05 compared to control rats; + p < 0.05 compared to non-treated diabetic rats.

Figure 5: Representative images for intraepidermal skin nerve fibers (A), sub-epithelial cornea nerve fibers as examined by corneal confocal microscopy (B), sub-epithelial nerve fibers in the region of the whorl examined in vitro using histochemical staining with β-anti-tubulin (C) and epithelial cornea nerve fibers examined in vitro using histochemical staining with β-anti-tubulin (D).
Table 1: Fatty Acid % Composition of Diets Measured by Gas Chromatography

<table>
<thead>
<tr>
<th>Diet</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:5</th>
<th>22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (3)</td>
<td>22 ± 3</td>
<td>2 ± 1</td>
<td>8 ± 1</td>
<td>28 ± 3</td>
<td>33 ± 4</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>25% Menhaden oil (3)</td>
<td>17 ± 3</td>
<td>13 ± 3</td>
<td>3 ± 1</td>
<td>12 ± 2</td>
<td>13 ± 2</td>
<td>13 ± 2</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E.M. ND: not detected. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid. Parentheses indicate the number of experimental determinations.
Table 2: Effect of Duration of Type 1 Diabetes in Sprague-Dawley Rats on Weight Gain, Blood Glucose, Hemoglobin A1C, Motor and Sensory Nerve Conduction Velocity, Thermal and Cornea Sensitivity, Intraepidermal Nerve Fiber Density and Cornea Nerve Fiber Density in the Sub-epithelial Layer

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control 4 week (6)</th>
<th>Diabetic 4 week (6)</th>
<th>Control 8 week (6)</th>
<th>Diabetic 8 week (6)</th>
<th>Control 12 week (6)</th>
<th>Diabetic 12 week (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start weight (g)</td>
<td>293 ± 5</td>
<td>300 ± 4</td>
<td>295 ± 3</td>
<td>306 ± 4</td>
<td>297 ± 3</td>
<td>296 ± 3</td>
</tr>
<tr>
<td>End weight (g)</td>
<td>364 ± 7</td>
<td>280 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>407 ± 7</td>
<td>300 ± 26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>437 ± 5</td>
<td>236 ± 14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>149 ± 8</td>
<td>558 ± 19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>154 ± 7</td>
<td>559 ± 29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>142 ± 8</td>
<td>572 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hb A1C (%)</td>
<td>7.8 ± 0.3</td>
<td>15.1 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9 ± 0.1</td>
<td>16.0 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7 ± 0.1</td>
<td>17.8 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MNCV (m/s)</td>
<td>51.1 ± 1.7</td>
<td>36.5 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.2 ± 2.6</td>
<td>38.0 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.0 ± 1.6</td>
<td>35.4 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SNCV (m/s)</td>
<td>33.4 ± 1.1</td>
<td>26.5 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.7 ± 0.9</td>
<td>26.0 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.2 ± 1.2</td>
<td>26.8 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thermal nociception (sec)</td>
<td>10.2 ± 0.4</td>
<td>9.8 ± 0.4</td>
<td>12.8 ± 0.3</td>
<td>18.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.9 ± 0.9</td>
<td>20.2 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intraepidermal nerve fibers</td>
<td>15.2 ± 0.9</td>
<td>17.2 ± 0.7</td>
<td>14.8 ± 0.7</td>
<td>16.1 ± 1.1</td>
<td>16.0 ± 0.7</td>
<td>13.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(profiles/mm)</td>
<td>15.2 ± 0.9</td>
<td>17.2 ± 0.7</td>
<td>14.8 ± 0.7</td>
<td>16.1 ± 1.1</td>
<td>16.0 ± 0.7</td>
<td>13.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cornea sensitivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cm)</td>
<td>5.9 ± 0.1</td>
<td>5.5 ± 0.2</td>
<td>5.8 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>5.9 ± 0.1</td>
<td>4.8 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cornea confocal microscopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mm/mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>7.8 ± 0.6</td>
<td>6.3 ± 0.7</td>
<td>8.0 ± 0.6</td>
<td>5.9 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.9 ± 0.8</td>
<td>5.0 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E.M. *p < 0.05 compared to control. The number of animals in each group is shown in parenthesis.
Table 3: Effect of Menhaden Oil Supplementation on Fatty Acid % Composition of Serum Measured by Gas Chromatography

<table>
<thead>
<tr>
<th>Diet</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:4</th>
<th>20:5</th>
<th>22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (13)</td>
<td>20.9 ± 1.5</td>
<td>16.1 ± 1.0</td>
<td>11.0 ± 0.4</td>
<td>22.1 ± 0.5</td>
<td>18.6 ± 0.4</td>
<td>0.4 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Control + menhaden oil (7)</td>
<td>21.9 ± 0.7</td>
<td>14.1 ± 0.6</td>
<td>6.3 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.6 ± 0.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>14.4 ± 0.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>10.9 ± 0.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8.3 ± 1.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic (10)</td>
<td>21.3 ± 0.8</td>
<td>19.6 ± 0.5</td>
<td>8.8 ± 0.3</td>
<td>24.1 ± 1.5</td>
<td>17.8 ± 1.9</td>
<td>0.1 ± 0.1</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Diabetic + menhaden oil prevention (9)</td>
<td>23.3 ± 0.5</td>
<td>12.3 ± 0.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>11.5 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.7 ± 0.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>10.1 ± 0.4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>8.9 ± 0.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>9.4 ± 0.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + menhaden oil intervention (9)</td>
<td>24.8 ± 0.5</td>
<td>12.7 ± 0.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>9.9 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.8 ± 0.7&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>10.1 ± 0.4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>7.4 ± 0.5&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>8.9 ± 0.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Fatty acid unsaturation index: Control 1.54 ± 0.04; Control + Menhaden oil 2.08 ± 0.01<sup>a,b</sup>; Diabetic 1.56 ± 0.02; Diabetic + Menhaden oil prevention 1.92 ± 0.06<sup>a,b</sup>; Diabetic + Menhaden oil intervention 1.88 ± 0.03<sup>a,b</sup>. Data are presented as the mean ± S.E.M. ND: not detected. <sup>a</sup>P < 0.05 compared to control; <sup>b</sup>P < 0.05 compared to non-treated diabetic; <sup>c</sup>P < 0.05 compared to control + menhaden oil. The number of animals in each group is shown in parenthesis.
Table 4: Effect of Menhaden Oil Dietary Enrichment in Streptozotocin Type 1 Diabetic Rats on Change in Body Weight, Blood Glucose, Hemoglobin A$_1C$ and Serum Triglycerides, Free Fatty Acids and Cholesterol

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control</th>
<th>Control + Menhaden oil</th>
<th>Diabetic</th>
<th>Diabetic + Menhaden oil Prevention</th>
<th>Diabetic + Menhaden oil Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(13)</td>
<td>(7)</td>
<td>(10)</td>
<td>(9)</td>
<td>(9)</td>
</tr>
<tr>
<td>Start weight (g)</td>
<td>298 ± 3</td>
<td>313 ± 5</td>
<td>307 ± 2</td>
<td>317 ± 3</td>
<td>317 ± 3</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>454 ± 9</td>
<td>475 ± 10</td>
<td>298 ± 19$^a$</td>
<td>323 ± 9$^a$</td>
<td>317 ± 13$^a$</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>154 ± 11</td>
<td>145 ± 5</td>
<td>543 ± 34$^a$</td>
<td>569 ± 19$^a$</td>
<td>496 ± 36$^a$</td>
</tr>
<tr>
<td>Hb A$_1C$ (%)</td>
<td>5.8 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td>16.3 ± 0.8$^a$</td>
<td>14.3 ± 1.0$^a$</td>
<td>17.9 ± 0.9$^a$</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>63 ± 7</td>
<td>44 ± 11</td>
<td>422 ± 55$^a$</td>
<td>370 ± 87$^a$</td>
<td>659 ± 121$^a$</td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.11 ± 0.01</td>
<td>0.30 ± 0.07</td>
<td>0.73 ± 0.14$^a$</td>
<td>0.73 ± 0.11$^a$</td>
<td>0.77 ± 0.07$^a$</td>
</tr>
<tr>
<td>Cholesterol (mg/ml)</td>
<td>0.90 ± 0.14</td>
<td>0.91 ± 0.06</td>
<td>3.73 ± 0.61$^a$</td>
<td>2.95 ± 0.71$^a$</td>
<td>5.29 ± 1.28$^a$</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E.M.  $^a$ P < 0.05 compared to control. Parentheses indicate the number of experimental animals.
Figure 1
Figure 2

Thermal nociception (sec)

Tactile response threshold (g)

Control    Control + MO    Diabetic    Diabetic + MO Prevention    Diabetic + MO Intervention

*  

+  

+  

+  

*  

*  

*  

*  

*  

*
Figure 3

![Bar chart showing IENF (profiles/mm) and Epithelial corneal nerve volume (%). The chart compares Control, Control + MO, Diabetic, Diabetic + MO Prevention, and Diabetic + MO Intervention groups. Significant differences are indicated by asterisks (*) and double asterisks (**) on the bars.]
Figure 4

Corneal nerve length (mm/mm²) vs. Corneal sensitivity (cm)

- Control
- Control + MO
- Diabetic
- Diabetic + MO Prevention
- Diabetic + MO Intervention

* indicates significant difference.
Figure 5

Intraepidermal nerve fibers

Corneal sub-epithelial nerves by CCM

Corneal sub-epithelial nerves in region of the whorl by standard confocal microscopy

Corneal epithelial nerves in region of the whorl by standard confocal microscopy