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

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However, the Diabetes Control and Complications Trial 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 (Coppey et al. 2012). In patients with type 2 diabetes, long-term treatments with eicosapentaenoic 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 preclinical 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 (St. Louis, MO).

Animals. Male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats 10–11 wk of age were housed in a certified animal care facility, and food (no. 7001; Harlan Teklad, Madison, WI) and water were provided ad libitum. All institutional (ACURF Approval No. 1290701) and National Institutes of Health 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 wk. For this study rats at 12 wk of age were separated into two groups. One of these groups was treated with streptozotocin (55 mg/kg in 0.1 M citric acid buffer, pH 4.5 ip). 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 U of Lantus insulin every other day to maintain body weight (Oltman et al. 2011). At 4, 8, and 12 wk, 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 wk of age were separated into five groups. Three of these groups were treated with streptozotocin, and diabetes was verified as described above. One group of diabetic rats (diabetic nontreated) remained on the standard diet for the entire 16 wk 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 wk and then was placed on the menhaden oil enriched diet for the final 8 wk 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-wk period.

Streptozotocin-induced type 1 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.

Peripheral neuropathy afflicts over 50% of patients with diabetes and is responsible for the majority of nontrauma-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).
of the study. The fatty acid compositions 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 heat 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 were turned off and the time was recorded. The timer was defaulted to go off after 25 s 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 seconds was 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 are reported in grams. Corneal sensation was measured using a Cochet-Bonnet filament esthesiometer (Luneau Ophthalmogie) 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 was repeated until a positive response was recorded. This process was repeated for each eye three times. The data are reported as centimeters.

**Motor and sensory nerve conduction velocity.** On the day of terminal studies, rats were weighed and anesthetized intraperitoneally with sodium pentobarbital (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 measured in seconds was 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).

**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 means ± SE. Parentheses indicate the number of experimental determinations. 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.
subepithelial corneal nerves was detected using corneal confo-
thermal hyperalgesia. In the cornea a significant decrease of
after 4 wk of diabetes, we did not observe any indication of
thermal hypoalgesia after 8 wk of hyperglycemia. In this study
with control rats after 4 wk of diabetes. Diabetic rats were
levels trended to increase. Both motor and sensory nerve
Table 2. Effect of duration of type 1 diabetes in Sprague-Dawley rats on weight gain, blood glucose, hemoglobin A\textsubscript{1C}, MNCV, SNCV, thermal and cornea sensitivity, intraepidermal nerve fiber density, and cornea nerve fiber density in the subepithelial layer

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control 4 wk (6)</th>
<th>Diabetic 4 wk (6)</th>
<th>Control 8 wk (6)</th>
<th>Diabetic 8 wk (6)</th>
<th>Control 12 wk (6)</th>
<th>Diabetic 12 wk (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*</td>
<td>407 ± 7</td>
<td>300 ± 26*</td>
<td>437 ± 5</td>
<td>236 ± 14*</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>149 ± 8</td>
<td>558 ± 19*</td>
<td>154 ± 7</td>
<td>559 ± 29*</td>
<td>142 ± 8</td>
<td>572 ± 17*</td>
</tr>
<tr>
<td>Hb A\textsubscript{1C}, %</td>
<td>7.8 ± 0.3</td>
<td>15.1 ± 0.7*</td>
<td>6.9 ± 0.1</td>
<td>16.0 ± 0.7*</td>
<td>5.7 ± 0.1</td>
<td>17.8 ± 1.1*</td>
</tr>
<tr>
<td>MNCV, m/s</td>
<td>51.1 ± 1.7</td>
<td>36.5 ± 2.0*</td>
<td>57.2 ± 2.6</td>
<td>38.0 ± 2.8*</td>
<td>52.0 ± 1.6</td>
<td>35.4 ± 2.6*</td>
</tr>
<tr>
<td>SNCV, m/s</td>
<td>33.4 ± 1.1</td>
<td>26.5 ± 0.8*</td>
<td>35.7 ± 0.9</td>
<td>26.0 ± 1.0*</td>
<td>34.2 ± 1.2</td>
<td>26.8 ± 1.2*</td>
</tr>
<tr>
<td>Thermal nociception, s</td>
<td>15.2 ± 0.4</td>
<td>9.8 ± 0.4</td>
<td>12.8 ± 0.3</td>
<td>18.4 ± 0.5*</td>
<td>9.9 ± 0.9</td>
<td>20.2 ± 1.3*</td>
</tr>
<tr>
<td>Intraepidermal nerve fibers, profiles/mm</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*</td>
</tr>
<tr>
<td>Cornea confocal microscopy, mm/mm\textsuperscript{2}</td>
<td>7.8 ± 0.6</td>
<td>6.3 ± 0.7</td>
<td>8.0 ± 0.6</td>
<td>5.9 ± 0.4*</td>
<td>7.9 ± 0.8</td>
<td>5.0 ± 0.7*</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SE. Number of animals in each group is shown in parenthesis. Hb A\textsubscript{1C}, hemoglobin A\textsubscript{1C}; MNCV and SNCV, motor and sensory nerve conduction velocity. *P < 0.05, compared with control.

RESULTS

Effect of type 1 diabetes duration of 4–12 wk on neuropathy. Table 2 presents data for the progression of diabetic neuropathy over the period of 4–12 wk after the induction of hyperglycemia in 12-wk-old rats. From 4 to 12 wk, hemoglobin A\textsubscript{1C} levels trended to increase. Both motor and sensory nerve conduction velocities were significantly decreased compared with control rats after 4 wk of diabetes. Diabetic rats were thermal hypoalgesic after 8 wk of hyperglycemia. In this study after 4 wk of diabetes, we did not observe any indication of thermal hyperalgesia. In the cornea a significant decrease of subepithelial corneal nerves was detected using corneal confocal microscopy after 8 wk of diabetes, and a significant decrease in cornea sensitivity was detected using a Cochet-Bonnet filament esthesiometer after 12 wk of diabetes. Based on these results we chose the time frame for the intervention protocol to be 8 wk of nontreated diabetes followed by 8 wk 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, nontreated diabetic rats, and diabetic rats treated with menhaden oil following a prevention or intervention protocol. Compared with serum from control rats, there is little change in the fatty acid composition in the serum from diabetic rats. Treating control rats for 16 wk 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 with 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 with control or nontreated diabetic rats. There was no difference in the fatty acid composition of serum in diabetic rats treated with menhaden oil for 8 wk (intervention) or 16 (prevention) wk. As expected the fatty acid unsaturation index was significantly increased in serum from diabetic rats treated with menhaden oil compared with control or nontreated diabetic rats (Table 3). The n-6 to n-3 fatty acid ratios in serum from control, control + menhaden oil, nontreated 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.

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*</td>
<td>12.6 ± 0.6*†</td>
<td>14.4 ± 0.4*†</td>
<td>10.9 ± 0.8*†</td>
<td>8.3 ± 1.0*†</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*†</td>
<td>11.5 ± 0.6</td>
<td>14.7 ± 0.9*†</td>
<td>10.1 ± 0.4*†</td>
<td>8.9 ± 0.6*†</td>
<td>9.4 ± 0.5*†</td>
</tr>
<tr>
<td>Diabetic + menhaden oil intervention (9)</td>
<td>24.8 ± 0.5</td>
<td>12.7 ± 0.9*†</td>
<td>9.9 ± 0.7</td>
<td>16.8 ± 0.7*†</td>
<td>10.1 ± 0.4*†</td>
<td>7.4 ± 0.5*†</td>
<td>8.9 ± 0.4*†</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SE. Number of animals in each group is shown in parenthesis. Fatty acid unsaturation index: control, 1.54 ± 0.04; control + menhaden oil, 2.08 ± 0.01*; diabetic, 1.56 ± 0.02; diabetic + menhaden oil prevention, 1.92 ± 0.06*; diabetic + menhaden oil intervention, 1.88 ± 0.03*; *P < 0.05, compared with control; †P < 0.0, compared with nontreated diabetic; ‡P < 0.05, compared with control + menhaden oil.

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Table 4.  Effect of menhaden oil dietary enrichment in streptozotocin type 1 diabetic rats on change in body weight, blood glucose, hemoglobin A	extsubscript{1C} and serum triglycerides, free fatty acids, and cholesterol

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control (13)</th>
<th>Control + Menhaden Oil (7)</th>
<th>Diabetic (10)</th>
<th>Diabetic + Menhaden Oil Prevention (9)</th>
<th>Diabetic + Menhaden Oil Intervention (9)</th>
</tr>
</thead>
<tbody>
<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*</td>
<td>323 ± 9*</td>
<td>317 ± 13*</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>154 ± 11</td>
<td>145 ± 5</td>
<td>543 ± 34*</td>
<td>569 ± 19*</td>
<td>496 ± 36*</td>
</tr>
<tr>
<td>Hb A	extsubscript{1C}, %</td>
<td>5.8 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td>16.3 ± 0.8*</td>
<td>14.3 ± 1.0*</td>
<td>17.9 ± 0.9*</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>63 ± 7</td>
<td>44 ± 11</td>
<td>422 ± 55*</td>
<td>370 ± 87*</td>
<td>659 ± 121*</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*</td>
<td>0.73 ± 0.11*</td>
<td>0.77 ± 0.07*</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*</td>
<td>2.95 ± 0.71*</td>
<td>5.29 ± 1.28*</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SE. Parentheses indicate the number of experimental animals. *P < 0.05, compared with control.

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 nontreated and treated diabetic rats failed to gain weight compared with 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 A	extsubscript{1C} values were significantly increased in nontreated 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 nontreated 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 in nontreated diabetic rats compared with control rats (Fig. 1). Treating diabetic rats using a prevention or intervention protocol with a high-fat diet enriched with menhaden oil significantly increased intraepidermal nerve fiber profiles compared with control rats. Treating diabetic rats with a diet enriched with menhaden oil using a prevention protocol significantly increased intraepidermal nerve fiber profiles compared with control rats. Treating control rats with a diet enriched with menhaden oil for 16 wk also significantly increased intraepidermal nerve fiber profiles compared with control rats.

Fig. 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. Data are presented as the means ± SE in m/s. The number of rats in each group was the same as shown in Table 3. *P < 0.05, compared with control rats; +P < 0.05, compared with nontreated diabetic rats.

Fig. 2. Effect of treatment of type 1 diabetic rats with MO-supplemented diet using a prevention or intervention protocol on thermal nociception and tactile response. Thermal sensitivity and tactile response threshold was significantly decreased in nontreated diabetic rats (Fig. 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 (Fig. 2).
Effect of type 1 diabetes and dietary treatment with menhaden oil on epithelial and subepithelial corneal nerve fibers and cornea sensitivity. Data in Figs. 3 and 4 demonstrate that corneal nerves of the subepithelial layer (Fig. 4) and epithelium (Fig. 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 (Fig. 4). Treating control rats with menhaden oil had no significant effect on corneal nerve density in the subepithelial layer or epithelium or on cornea sensitivity. Figure 5 provides representative images of intraepidermal nerve fibers in the skin from a hindpaw (top left), subepithelial corneal nerves obtained using corneal confocal microscopy (top right), subepithelial 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 (bottom left) 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 (bottom right). All images were obtained from a control animal.

FIGURE 3. Effect of treatment of type 1 diabetic rats with MO-supplemented diet using a prevention or intervention protocol on intraepidermal nerve fiber (IENF) and epithelial corneal nerve density. Intraepidermal and epithelial corneal nerve fiber density was determined as described in MATERIALS AND METHODS. Data are presented as the means ± SE in profiles/mm² for intraepidermal nerve fiber density and area percent 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 with control rats; +P < 0.05, compared with nontreated diabetic rats.

Effect of treatment of type 1 diabetic rats with a diet enriched with menhaden oil on subepithelial corneal nerve fiber length and cornea sensitivity. Data in Figs. 3 and 4 demonstrate that subepithelial corneal nerve fiber length and area percent 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 with control rats; +P < 0.05, compared with nontreated diabetic rats. 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 wk of nontreated diabetes. Reduction in motor and sensory nerve conduction velocity was the first deficit to appear after 4 wk of diabetes. Impairment of functional and structural deficits in the skin and cornea occurred after 8–12 wk 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 nontreated diabetic rats. More importantly, intervention after 8 wk of nontreated diabetes with menhaden oil enriched diet reversed the neuropathological changes after only 8 wk of treatment.

The serum fatty acid profile was not significantly different between control and diabetic rats. However, after 8–16 wk of treatment with menhaden oil both control and diabetic rats had a significantly different serum fatty acid profile compared with 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 with untreated control rats and a significant decrease in stearic, linoleic, and arachidonic acids in diabetic rats treated with menhaden oil compared with untreated diabetic rats. Notable differences 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 determinations of nerve conduction velocities are standard endpoints for the study of diabetic neuropathy, whereas examinations 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).

FIGURE 4. Effect of treatment of type 1 diabetic rats with MO-supplemented diet on subepithelial cornea nerve fiber length and cornea sensitivity. Subepithelial cornea nerve fiber length and cornea sensitivity was determined as described in MATERIALS AND METHODS. Data are presented as the means ± SE in mm/mm² for subepithelial 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 with control rats; +P < 0.05, compared with nontreated diabetic rats.
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 with control rats treated with menhaden oil and levels of arachidonic acid in control rats treated with menhaden oil were significantly higher compared with diabetic rats treated with menhaden oil. The reason for these differences is 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/n-3 fatty acid ratio. Lowering of the n-6/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 is significantly reduced in diabetic rats treated with 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, 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. 2012). 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 vasodilatation 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 epi neural 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 antioxidant, antiinflammatory, and neuroprotection 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 nonvascular tissue, 15-lipoxygenase-1 is responsible for the generation of resolvins and neuroprotecin 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 type 1 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.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

DISCLAIMER

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.

AUTHOR CONTRIBUTIONS

Author contributions: I.J.C., E.P.D., A.O., and M.A.Y. performed experiments; I.J.C., E.P.D., A.O., and M.A.Y. analyzed data; I.J.C., E.P.D., A.O., and M.A.Y. approved final version of manuscript; E.P.D. and M.A.Y. prepared figures; M.A.Y. conception and design of research; M.A.Y. interpreted results of experiments; M.A.Y. drafted manuscript; M.A.Y. edited and revised manuscript.

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