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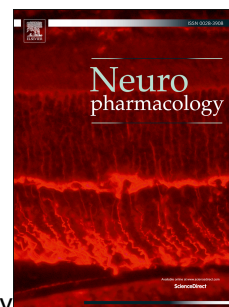
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Early vs. late intervention of high fat/low dose streptozotocin treated C57Bl/6J mice with enalapril, α -lipoic acid, menhaden oil or their combination: effect on diabetic neuropathy related endpoints

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Abstract

We have previously demonstrated that enalapril, α -lipoic acid and menhaden (fish) oil has potential as a treatment for diabetic peripheral neuropathy. In this study we sought to determine the efficacy of these treatments individually or in combination on multiple neuropathic endpoints in a high fat fed low dose streptozotocin treated mouse, a model of type 2 diabetes, following early or late intervention. Four or twelve weeks after the onset of hyperglycemia, diabetic mice were treated with enalapril, α -lipoic acid, menhaden oil or their combination for 12 weeks. Afterwards, endpoints including glucose tolerance, motor and sensory nerve conduction velocity, thermal nociception, and intraepidermal and cornea nerve fiber density was determined. Glucose clearance was impaired in diabetic mice and significantly improved only with combination treatment and early intervention. Diabetes caused steatosis, slowing of motor and sensory nerve conduction velocity, thermal hypoalgesia and reduction in intraepidermal and cornea nerve fiber density. Treating diabetic mice with enalapril, α -lipoic acid or menhaden oil partially protected diabetic mice from these deficits, whereas the combination of these three treatments was more efficacious following early or late intervention. These studies suggest that a combination therapy may be more effective for treating neural complications of type 2 diabetes.

Key words: diabetic peripheral neuropathy; type 2 diabetes; enalapril; α -lipoic acid; menhaden oil; corneal nerves

Chemical compounds: Enalapril maleate (PubChem CID: 5388961); α -lipoic acid (PubChem CID: 864)

Highlights

- Menhaden (fish) oil, α -lipoic acid and enalapril singularly can improve diabetic peripheral neuropathy.
- Combination therapy is more effective than monotherapy in treating diabetic peripheral neuropathy.
- Diabetic peripheral neuropathy is treatable even after chronic hyperglycemia.
- Diabetes-induced loss of intraepidermal nerve fibers and sub-epithelial corneal nerve fibers is reversible.

1. Introduction

Peripheral neuropathy is the most common complication of diabetes mellitus and has also been reported to occur in animal models and humans regarded as being pre-diabetic (Cortez et al., 2014; Davidson et al., 2014; Papanas and Ziegler, 2012; Singh et al., 2014; Singleton and Smith, 2007; Vinik et al., 2013; Ziegler et al., 2014a). Studies using rodent models of diabetes have been successful in identifying mechanisms and possible treatments for diabetic peripheral neuropathy, but translation of these findings to humans has failed (Albers and Pop-Busui, 2014; Obrosova, 2009; Singh et al., 2014; Vincent et al., 2011; Yagihashi et al., 2011; Yorek, 2008; Zychowska et al., 2013). Thus far most clinical studies for treatment of diabetic peripheral neuropathy have focused on using mono-therapeutic treatments believing that intervening at a single pathological mechanism will successfully abate this disease. However, diabetic peripheral neuropathy is a complex disease with multiple mechanisms contributing to its etiology and it is unlikely that a mono-therapeutic approach will be maximally successful (Albers and Pop-Busui, 2014; Singh et al., 2014; Vincent et al., 2013; Yagihashi et al., 2011; Yorek, 2008). Furthermore, little is known to what extent an early vs. late intervention can be successful in treatment of diabetic peripheral neuropathy. Studies have been ongoing to identify early marker(s) of peripheral neuropathy (Arimura et al., 2013; Divisova et al., 2016; Malik et al., 2011; Papanas and Ziegler 2012; 2013; Pritchard et al., 2011; Ziegler et al., 2014b). As more effective bio-markers for peripheral neuropathy become available, studies need to go forward to identify possible new interventions that can be applied safely in clinical trials. In this regard we have focused on examining the use of a combination of dietary supplements and drugs (enalapril, α -lipoic acid and menhaden [fish] oil) for treatment of diabetic peripheral neuropathy in rodent models (Davidson et al., 2014). The reason for selecting these three compounds is that we have demonstrated that each of these treatments individually had beneficial effects on diabetic peripheral neuropathy and each have also been shown to be safe for use in humans (Coppey et al., 2001; 2006; 2012; 2015; Davidson et al., 2011; Gopinath et

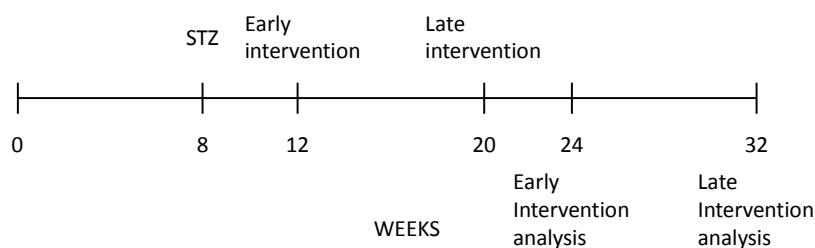
al., 2012; McIllduff and Rutkove, 2011; Papanas and Ziegler, 2014; Rudkowska, 2010; Shevalye et al., 2015; Yee et al., 2010; Ziegler et al., 2011). The primary mechanism of action of these three compounds is oxidative and inflammatory stress. The study design examined the effect of treatments following an early or late intervention I on development and progression of diabetic peripheral neuropathy using multiple endpoints in a mouse model of type 2 diabetes, that we had previously characterized (Yorek et al., 2015).

2. Materials and Methods

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

2.2 *Animals:* C57Bl6/J male mice were purchased from Jackson Laboratories. Mice were housed in a certified animal care facility with standard diet (Harlan Teklad, #7001, Madison, WI) and water provided ad libitum. All experiments were conducted in accordance with international standards on animal welfare and were compliant with institutional and National Institutes of Health guidelines for use of animals (ACORP protocol 1390201). Two separate experiments (early and late intervention design) were conducted (see graphical timeline below). For the early intervention study mice at 12 weeks of age were divided into six groups. After 1 week on a standard diet (Harlan Teklad, #7001; Madison, WI) five of the groups were fed a 60 kcal% high fat diet (D12492; Research Diets, New Brunswick, NJ). The other group (control) remained on the standard diet for the duration of the study. For the late intervention study mice were divided into eight groups and six groups were fed the 60 kcal% high fat diet while the other two groups remained on the standard diet. To create the type 2 diabetic model the high fat fed mice, after 8 weeks on the high fat diet, were treated with 75 mg/kg streptozotocin (EMD Chemicals, San Diego, CA) followed three days later with a second dose of streptozotocin (50 mg/kg) as needed. Mice with blood glucose ≥ 13.8 mM (250 mg/dl) were considered diabetic. These groups remained on the high fat diet for an additional four weeks for the early intervention study and twelve weeks for the late intervention study. Afterwards, one group designated as the untreated diabetic group in each of the early and late intervention studies remained on the 60 kcal% high fat diet for the entire period of the study. The other four groups received the 60 kcal% high fat diet containing: menhaden oil ($\frac{1}{2}$ of the fat [derived primarily from lard] in the 60 kcal% high fat diet was substituted with menhaden oil), or enalapril (500 mg/kg in the 60 kcal% high fat diet; BOC Sciences, Shirley, NY), or α -lipoic acid (2.5 g/kg in the 60 kcal% high fat diet) or the combination of all three. The modified diets were prepared by Research

Diets. The doses of the agents used in the modified diets were based on our previous studies (Coppey et al., 2001; 2006; 2012; Davidson et al., 2015). The base diet was the same for all the diabetic mice in these studies and we found that each group ate about the same amount of chow throughout the study (~ 75 g/kg mouse). These diets (treatment phase) were maintained for 12 weeks for both the early and late intervention. For the late intervention study, a group of control and untreated diabetic mice were studied at the time treatment began to establish a baseline for the pathology present after 12 weeks of untreated hyperglycemia. All studies were performed in a masked fashion by providing a coding for each animal that was unknown by the examiner.



2.3 *Intraperitoneal glucose tolerance test:* After 12 weeks of diet intervention intraperitoneal glucose tolerance test was performed after an overnight fast as previously described (Coppey et al., 2011).

2.4 *Behavioral test:* Immediately prior to the terminal studies thermal nociceptive response in the hindpaw was measured using the Hargreaves method as previously described (Coppey et al., 2011).

2.5 *Motor and sensory nerve conduction velocity:* On the day of terminal studies mice were weighed and anesthetized with sodium pentobarbital (75 mg/kg, i.p., Diamondback Drugs, Scottsdale, AZ). Non-fasting blood glucose was determined. Motor and sensory nerve conduction velocities were determined as previously described (Coppey et al., 2011).

2.6 *Corneal innervation in vivo:* Sub-epithelial corneal nerves were imaged in vivo non-invasively using the Rostock cornea module of the Heidelberg Retina Tomograph

(Heidelberg Engineering, Vista, CA) confocal microscope as previously described (Yorek et al., 2014; 2015).

2.7 Immunohistochemistry analysis of nerves in the skin and cornea in vitro: After completion of all *in vivo* analyses skin from the footpad and corneas were collected. These tissues were fixed and analyzed for presence of intraepidermal nerve fibers and corneal nerves (sub-epithelial and nerves penetrating the epithelium), respectively, as previously described (Yorek et al., 2014; 2015).

2.8 Analyses in liver and serum: To examine steatosis, liver samples were frozen in OCT compound (Sakura FineTek USA, Torrance, CA) in liquid nitrogen. Liver sections, 5 μ m, were incubated with BODIPY (Molecular Probes, Carlsbad, CA), at a 1:5,000 dilution in 1% bovine serum albumin for 1h at room temperature. After washing liver sections were mounted using ProLong® Gold antifade reagent (Molecular Probes, Carlsbad, CA) and covered with a glass coverslip. Images were collected using Zeiss 710 LSM confocal laser scanning microscope. Images were analyzed for % area fraction of lipid droplets using Image J software (Shevalye et al., 2015). Protein bound 3-nitrotyrosine concentration was measured in liver by indirect enzyme-linked immunosorbent assay as described (Weber et al., 2012) and modified by (Yorek et al., 2014). Serum was used for determining levels of free fatty acid, triglyceride and free cholesterol using commercial kits from Roche Diagnostics, Mannheim, Germany; Sigma Aldrich Chemical Co., St. Louis, MO; and BioVision, Mountain View, CA, respectively (Yorek et al., 2014).

2.9 Data analysis: Results are presented as mean \pm SEM. Comparisons between groups were conducted using a one-way ANOVA and Bonferroni's test for multiple comparisons (Prism software; GraphPad, San Diego, CA). A p value of less than 0.05 was considered significant.

3. Results

3.1 *Early Intervention:*

3.1.1 Metabolic parameters: Data in Table 1 show that all mice at the beginning of the study weighed approximately the same. After 20 weeks of a high fat diet combined with 16 weeks of hyperglycemia untreated diabetic mice weighed significantly more than age-matched control mice. Treating diabetic mice for 12 weeks with a high fat diet enriched with menhaden oil or supplemented with α -lipoic acid did not affect their final weight. In contrast, diabetic mice treated with a high fat diet supplemented with enalapril or with the combination of all three compounds reduced weight gain and these mice weighed about the same as control mice at the end of the study. Untreated diabetic mice and diabetic mice treated with a high fat diet enriched with menhaden oil or supplemented with α -lipoic acid or enalapril were hyperglycemic as indicated by increased non-fasting blood glucose levels. However, blood glucose levels were significantly improved compared to untreated diabetic mice when diabetic mice were treated with the combination of menhaden oil, α -lipoic acid and enalapril in the high fat diet. Data in Figure 1 (top) demonstrate that glucose clearance is significantly impaired in untreated diabetic mice and diabetic mice fed a high fat diet enriched with menhaden oil or supplemented with α -lipoic acid or enalapril. Treating diabetic mice with the high fat diet containing the combination of menhaden oil, α -lipoic acid and enalapril significantly improved glucose clearance compared to untreated diabetic mice but glucose clearance remained significantly impaired compared to control mice. As noted in the legend of Figure 1, fasting blood glucose was significantly increased in untreated diabetic mice and diabetic mice treated with the high fat diet enriched with menhaden oil or supplemented with α -lipoic acid. However, treating diabetic mice with the high fat diet supplemented with enalapril or to a greater extent with the combination of menhaden oil, α -lipoic acid and enalapril significantly decreased fasting blood glucose levels compared to untreated diabetic mice.

Data in Table 1 also demonstrate that serum triglyceride levels are significantly increased in untreated diabetic mice and diabetic mice treated with the high fat diet enriched with menhaden oil or supplemented with α -lipoic acid or enalapril. In contrast, serum triglyceride levels in diabetic mice treated with the combination of menhaden oil, α -lipoic acid and enalapril were lower than serum triglyceride levels in control mice. Serum free fatty acid levels in untreated or treated diabetic mice were not different from control mice except for diabetic mice treated with the combination of menhaden oil, α -lipoic acid and enalapril. In these mice serum free fatty acid levels were significantly lower than control and untreated diabetic mice. Serum cholesterol levels were significantly increased in untreated diabetic mice compared to control mice and mono-therapy or the combination of menhaden oil, α -lipoic acid and enalapril normalized serum cholesterol levels. Liver nitrotyrosine, a marker for oxidative stress, levels were significantly increased in untreated diabetic mice and diabetic mice treated with α -lipoic acid or enalapril compared to control mice. Liver nitrotyrosine levels in diabetic mice treated with the high fat diet enriched with menhaden oil were significantly higher than untreated diabetic mice and control mice. Since mono-therapy treatments alone had no effect on improving liver nitrotyrosine levels it was surprising to find that treating diabetic mice with the combination of menhaden oil, α -lipoic acid and enalapril in the high fat diet significantly improved liver nitrotyrosine levels compared to untreated diabetic mice. Untreated diabetic mice had a high degree of fatty liver and treatment of diabetic mice with a high fat diet enriched in menhaden oil or supplemented with α -lipoic acid or to a greater extent enalapril trended to improve steatosis but the liver lipid droplet content of all mono therapy treated mice remained significantly elevated compared to control mice. In contrast, treating diabetic mice with the high fat diet containing the combination of menhaden oil, α -lipoic acid and enalapril completely prevented steatosis.

3.1.2 Neurology and corneal morphology: We next examined multiple neuropathic endpoints associated with diabetic peripheral neuropathy. Both motor and sensory nerve

conduction velocities were significantly decreased in untreated diabetic mice (Table 2). All treatments significantly improved motor nerve conduction velocity. Menhaden oil enrichment of the high fat diet or the combination therapy tended to provide the greatest benefit but there was no statistical difference between the beneficial effects of any of the treatments. Likewise, treating diabetic mice with a high fat diet enriched with menhaden oil or the combination therapy provided the greatest improvement in sensory nerve conduction velocity. Treating diabetic mice with the high fat diet containing α -lipoic acid or enalapril also significantly improved sensory nerve conduction velocity compared to untreated diabetic mice but it remained significantly decreased compared to control mice.

Response to a thermal stimulus applied to the hindpaw was significantly impaired in untreated diabetic mice compared to control mice (Table 2). There was also a significant decrease in intraepidermal nerve fiber profiles in the skin of the hindpaw of untreated diabetic mice compared to control mice (Table 2). Treating diabetic mice by enriching the high fat diet with menhaden oil or supplementing it with α -lipoic acid or enalapril or providing diabetic mice with all three treatments combined significantly improved thermal nociception compared to untreated diabetic mice but each remained impaired when compared to control mice. A similar trend was observed of the effect of mono-therapy treatments on skin intraepidermal nerve fiber profiles. Combination therapy provided the greatest benefit for protecting intraepidermal nerve fiber density in the skin of diabetic mice.

Figure 2 provides representative images from a control mouse of sub-epithelial corneal nerves acquired using corneal confocal microscopy (left) and corneal nerves of the sub-epithelial layer (middle) and corneal nerves penetrating the cornea epithelium (right) following collection and fixation of the cornea and immunostaining using anti-tubulin III. Collection of these images from all mice was used to generate the cumulative data provided in Figures 3 and 4. Data in Figure 3 provides results for the effect of diabetes and treatment on density of cornea nerve fibers in the sub-epithelial layer using *in vivo* and *in vitro* methodology and in the corneal

epithelium. Untreated diabetes caused a significant decrease in corneal nerves in the sub-epithelial layer as determined *in vivo* using corneal confocal microscopy (Figure 3 left) or *in vitro* by immunostaining the cornea using anti-tubulin III (Figure 3 middle). Untreated diabetes also caused a significant decrease in corneal nerves penetrating the epithelium (Figure 3 right). Treating diabetic mice by supplementing the high fat diet with α -lipoic acid provided the least benefit toward protecting corneal nerve density in the sub-epithelial layer. Mono-therapy by enriching the high fat diet with menhaden oil or supplementing with enalapril provided a greater benefit of protecting corneal nerves of the sub-epithelial layer than α -lipoic acid in diabetic mice. Mono-therapy of menhaden oil or α -lipoic acid provided no significant improvement compared to control and untreated diabetic mice in protecting corneal nerves penetrating the cornea epithelium (Figure 3 right). In contrast, treating diabetic mice with enalapril alone or to a greater extent combination therapy of menhaden oil, α -lipoic acid and enalapril provided the greatest benefit with density of corneal nerves in the epithelium being preserved at levels seen in control mice.

3.2 Late Intervention:

3.2.1 Metabolic parameters: While the early intervention (4 weeks after the induction of hyperglycemia) study allowed for the determination whether mono- or combination therapy could slow neuropathic progression in a diabetic type 2 mouse model, the following studies examined whether the same treatments applied later (12 weeks after the induction of hyperglycemia) could induce reversal of neuropathology associated with chronic untreated hyperglycemia.

The first two columns in Tables 3 and 4 provide data of the status of control and diabetic mice at the time treatment was initiated. Previously we reported that 12 weeks of untreated diabetes resulted in significant impairment of multiple neural endpoints (Yorek et al., 2015). At the time of treatment diabetic mice weighed significantly more than control mice, were hyperglycemic, had elevated levels of serum triglycerides, cholesterol, and nitrotyrosine and had

fatty livers (Table 3). Changes in these mice directly related to diabetic neuropathy included reduced motor and sensory nerve conduction velocity, thermal hypoalgesia and decreased intraepidermal nerve fibers (Table 4). After an additional 12 weeks of no treatment these changes in diabetic mice compared to control mice persisted (Table 4). Treating diabetic mice with enalapril or the combination of menhaden oil, α -lipoic acid and enalapril caused a significant reduction in body weight compared to untreated diabetic mice (Table 3). Blood glucose levels at the end of the study improved in diabetic mice treated with the mono-therapies of menhaden oil or α -lipoic acid or the combination therapy. However, there was no significant improvement in glucose clearance in the mono- or combination therapy treated diabetic mice compared to untreated diabetic mice (Figure 1 bottom). Serum triglyceride levels were significantly improved/reversed in diabetic mice treated with mono-therapy of menhaden oil, α -lipoic acid or enalapril (Table 3). The greatest improvement in serum triglyceride levels was observed in mice treated with combination therapy. Serum free fatty acid levels were significantly decreased in diabetic mice treated with menhaden oil or enalapril or the combination therapy. Unlike early intervention treatment of diabetic mice, treatment of diabetic mice after chronic hyperglycemia did not significantly improve serum cholesterol levels. Liver nitrotyrosine levels were improved following treatment with α -lipoic acid, enalapril or combination therapy. Liver steatosis in untreated diabetic mice was increased following 12 additional weeks of hyperglycemia. Treating chronically hyperglycemic mice with menhaden oil, α -lipoic acid or enalapril significantly decreased steatosis compared to untreated diabetic mice but the degree of fatty liver remained significantly elevated compared to control mice. However, treating chronically hyperglycemic diabetic mice with the combination of menhaden oil, α -lipoic acid and enalapril completely reversed liver steatosis.

3.2.2 Neurology and corneal morphology: Data in Table 4 demonstrate that treating chronically hyperglycemic mice with menhaden oil or combination therapy completely reversed slowing of motor nerve conduction velocity. Slowing of motor nerve conduction velocity was

also improved with α -lipoic acid and enalapril treatment. Slowing of sensory nerve conduction velocity was significantly improved with mono- or combination therapy compared to chronically hyperglycemic mice. However, comparing data for sensory nerve conduction velocity of control and diabetic mice at time of treatment to the end of the study, sensory nerve conduction velocity was slower in both control and untreated diabetic mice apparently due to aging. Overall, treating diabetic mice with mono- or combination therapy appeared to halt progression of impairment. Treating chronically hyperglycemic diabetic mice with mono- or combination therapy reversed deficits in thermal hypoalgesia and intraepidermal nerve fiber density. However, both thermal nociception and intraepidermal nerve fiber density remained significantly impaired compared to control mice following mono- or combination therapy of diabetic mice.

Data in Figure 4 demonstrate that treating chronically hyperglycemic mice with menhaden oil, enalapril or the combination of menhaden oil, α -lipoic acid and enalapril can reverse loss of corneal nerve fibers in the sub-epithelial layer (Figure 4 left and middle) and penetrating the epithelium (Figure 4 right). At time of treatment sub-epithelial corneal nerves as assessed by corneal confocal microscopy was 3.6 ± 0.3 and $1.3 \pm 0.2^*$ mm/mm² for control and diabetic mice, respectively (* $p < 0.05$ compared to control). Treating diabetic mice with α -lipoic acid alone was less effective than treatment with menhaden oil or enalapril alone (Figure 4 left). Combination therapy was the most effective in reversing loss of corneal nerves in the sub-epithelial layer as assessed by corneal confocal microscopy. Similar results were obtained when sub-epithelial corneal nerve density was assessed by immunohistochemistry (Figure 4 middle). At the time of treatment corneal nerve fiber density of the sub-epithelium, as determined by immunohistochemistry, was 56.9 ± 1.4 and $48.4 \pm 2.3^*$ % for control and diabetic mice, respectively (* $p < 0.05$ compared to control). Corneal confocal microscopy in vivo is unable to image or assess the loss of corneal nerves penetrating the epithelium. We have previously demonstrated that these nerves are decreased early in diabetes (Davidson et al.,

2012a). At the time of treatment corneal nerve fiber density in the epithelium as determined by immunohistochemistry was 1.21 ± 0.04 and $0.88 \pm 0.06^*$ % for control and diabetic mice, respectively (* $p < 0.05$ compared to control). Data in Figure 4 (right) demonstrate that treatment of diabetic mice with α -lipoic acid alone halted/delayed progression of loss of corneal nerves penetrating the epithelium. In contrast, mono-therapy with menhaden oil or enalapril significantly improved/reversed corneal nerve fiber density in the epithelium. Combination therapy was most effective in improving/reversing nerve fiber density in the epithelium.

4. Discussion

Since the etiology of diabetic peripheral neuropathy is widely believed to be due to multiple mechanisms it should not be surprising that the mono-therapy approach taken in clinical trials to treat human diabetic subjects with clinical signs of peripheral neuropathy has not been successful. It is common for clinicians to mask or relieve pain of human subjects with painful diabetic neuropathy by prescribing two or more medications (Javed et al., 2015; Vinik et al., 2013). Also, other complicated human diseases with multiple etiologies such as hypertension are also commonly treated with two or more medications (James et al., 2014). Therefore, the concept that diabetic peripheral neuropathy can be treated with a singular therapeutic approach is not realistic. We have taken the approach that the most likely successful treatment for diabetic peripheral neuropathy that could easily be translated to human subjects would be a combination of agents that have singularly been shown to have beneficial effects on diabetic peripheral neuropathy in pre-clinical studies and have a proven safety record in human subjects (Davidson et al., 2015). In this manuscript we report on the effect of menhaden oil, α -lipoic acid, or enalapril or their combination following early or late intervention on multiple endpoints of diabetic peripheral neuropathy in a mouse model of type 2 diabetes (Yorek et al., 2015). The studies presented here in mice confirm in a second species that combination therapy is more effective than monotherapy for treatment of diabetic peripheral neuropathy and that even late intervention can induce reversal of end points associated with diabetic peripheral neuropathy (Davidson et al., 2015). These points are significant. Confirming similar outcomes in two different species provides greater rationale for advancing toward clinical trials. Moreover, most pre-clinical studies have focused on a prevention or early intervention protocol. In this study we compared the impact of treatment following 4 (early) or 12 (late) weeks of hyperglycemia. At 4 weeks of hyperglycemia the only significant deficit related to diabetic neuropathy is slowing of nerve conduction velocity. After 12 weeks of hyperglycemia we have shown that many end points associated with peripheral neuropathy are impaired

including nerve conduction velocity, thermal nociception, loss of intraepidermal nerve fibers in the skin and loss of corneal nerve fibers in the sub-epithelial layer and penetrating the epithelium (Yorek et al., 2015).

The type 2 diabetes mouse model for this study has been widely used. Generally this mouse models late stage type 2 diabetes. High fat fed C57Bl6/J mice which have higher circulating insulin levels and low to moderate hyperglycemia tend to model an earlier stage of type 2 diabetes (Chen et al., 2014; Mali et al., 2014; Yorek et al., 2015). The high fat fed low-dose streptozotocin treated mice have a much higher blood glucose level than high fat fed mice and circulating insulin levels are similar to control mice (Yorek et al., 2015). In contrast to type 1 diabetic mouse, which often lose weight compared to control mice, these type 2 diabetic mice weigh more than normal chow fed mice (Yorek et al., 2015).

We examined the influence of three treatments in this study. Enalapril is an angiotensin converting enzyme inhibitor and these inhibitors have been a widely prescribed treatment for hypertension and diabetic nephropathy (Barnett, 2006; Davidson et al., 2012b; Hilleman, 2000; Malik et al., 1998; Mogensen, 2005; Ruggenenti et al., 2010; Smith, 2002). Malik, et al. (1998) demonstrated that the angiotensin converting enzyme inhibitor trandolapril improved peripheral neuropathy in normotensive patients with diabetes. In studies with diabetic rats we and others have shown that inhibition of angiotensin converting enzyme can improve diabetic peripheral neuropathy (Aggarwal et al., 2001; Cameron et al., 1993; Coppey et al., 2006; Manschot et al., 2003). α -Lipoic acid, also known as thioctic acid, is an organosulfur compound derived from octanoic acid. α -Lipoic acid is made in mammals, including humans, naturally, and is essential for aerobic metabolism. It is also manufactured and is available as an over the counter dietary supplement as an antioxidant. After a four year study of treatment for mild-to-moderate diabetic distal symmetric sensorimotor polyneuropathy, α -lipoic acid did not influence the primary composite end point but resulted in a clinically meaningful improvement and prevention of progression of neuropathic impairments and was well tolerated (Ziegler et al., 2011). Menhaden

oil is derived from the Menhaden, a forage fish of the genera *Brevoortia* *Ethmidium* that resides in Atlantic, Pacific and Gulf waters. Oil prepared from these fish is commonly used as a source for omega-3 (n-3) polyunsaturated fatty acids, enriched in eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids for human consumption as an over the counter dietary supplement. Fish oil supplements have been used for cardiovascular disease and have been shown to improve metabolic features associated with type 2 diabetes (De Leiris et al., 2009; Jelinek et al., 2013; Mozaffarian and Wu, 2011).

In this study, like in diabetic rats, using an early intervention protocol, we found that combining enalapril, α -lipoic acid and menhaden oil improved neuropathy deficits overall to a greater extent than mono-therapy (Davidson et al., 2015). More importantly in this study we found that late intervention with combination therapy was successful in reversing diabetic neuropathy end points. The effect of these treatments on outcome was not due to the mice consuming different amounts of the diet. The base diet for the non-treated mice and for all of the treatments was 60 kcal% high fat diets. We found that each individual group of diabetic mice consumed about the same amount of chow. In some cases, such as thermal nociception and intraepidermal nerve fiber density, we found that impairment was significantly improved/reversed in diabetic mice with treatment but the deficits were not completely prevented. In contrast, combination therapy completely reversed deficits after early or late intervention for motor nerve conduction velocity and hepatic steatosis. For sensory nerve conduction velocity combination therapy significantly reversed deficits but did not fully restore nerve function. Unlike early intervention with combination therapy mono-therapies had no effect on improving glucose clearance. However, with late intervention no treatment was found to improve glucose clearance. The combination therapy also reduced diabetes induced blood glucose levels in both early and late intervention protocols.

Loss of corneal nerves in the sub-epithelial layer as detected by non-invasive corneal confocal microscopy is being promoted as a means of early detection of diabetic peripheral

neuropathy in humans as well as in pre-clinical studies (Davidson et al., 2012a; Malik et al., 2011; Papanas and Ziegler, 2013; Pritchard et al., 2011; Ziegler et al., 2014b). In this study we found that early intervention with mono-therapies and to a greater extent combination therapy was able to slow progression of loss of corneal nerves in the sub-epithelial layer as well as the corneal nerves penetrating the epithelium. With late intervention we found that combination therapy and to a lesser extent mono-therapy was able to reverse loss of corneal nerves and promote regeneration. Therefore, this study demonstrated that mono-therapy of enalapril, α -lipoic acid and menhaden oil and to a greater extent the combination of all three treatments was able to reverse some neuropathic endpoints as well as steatosis and at the very least slow progression of loss of sensory nerves in the skin and cornea after early intervention. At the time of late intervention all measured end points associated with diabetic peripheral neuropathy were impaired and combination therapy and to some extent mono-therapy improved/reversed these deficits.

Mechanisms contributing to neural dysfunction in diabetes that are improved by enalapril, α -lipoic acid or menhaden oil would likely be oxidative and inflammatory stress; it is known that increased production of metabolites of n-3 polyunsaturated fatty acids have anti-inflammatory and neuro-protective properties (Ji et al., 2011; Kohli and Levy, 2009; Serhan et al., 2002). We have previously shown that enalapril and α -lipoic acid reduce markers of oxidative stress in blood vessels and serum of diabetic rats (Coppey et al., 2001; 2006; Davidson et al., 2011; 2012b; Oltman et al., 2008; Yorek, 2008). We have also shown that enriching diets of diabetic rodents with menhaden oil reduces the n-6 to n-3 fatty acid ratio and increases production of the docosahexaenoic metabolite resolvin D1 (Coppey et al., 2012; 2015; Shevalye et al., 2015). Additionally, we have shown that direct treatment of a mouse model of type 2 diabetes with daily injections of resolvin D1 improved diabetic peripheral neuropathy including intraepidermal nerve fiber density and density of sub-epithelial corneal nerve fibers (Shevalye et al., 2015). In this study combination therapy and early intervention

was more effective than mono-therapy in reducing liver nitrotyrosine levels. With late intervention liver nitrotyrosine levels were corrected with treatment by α -lipoic acid, enalapril and combination therapy. Interestingly, mono-therapy treatment of diabetic mice with menhaden oil increased liver nitrotyrosine levels but when treatment included α -lipoic acid and enalapril this independent effect of menhaden oil did not occur. These results suggest that combination therapy of enalapril, α -lipoic acid and menhaden oil would likely be effective in reducing oxidative and inflammatory stress.

Pre-clinical testing of the effect of combination therapies for diabetic neuropathy has been earlier attempted by other laboratories as well as ours. We earlier demonstrated that combining α -lipoic acid with fidarestat, an aldose reductase inhibitor, was more efficacious than either treatment alone on diabetic peripheral neuropathy (Yorek et al., 2004). Using a vasopeptidase inhibitor, Ilexatril, a combination drug consisting of an inhibitor of angiotensin converting enzyme and neutral endopeptidase, we have shown the combination of these two drugs to be more effective than an angiotensin converting enzyme or neutral endopeptidase inhibitor alone (Davidson et al., 2012b). Other combination therapies that have been tested pre-clinically for diabetic peripheral neuropathy in rodent models include melatonin and nicotinamide which demonstrated that simultaneous inhibition of oxidative stress and activation of poly (ADP-ribose) polymerase may be a useful pharmacotherapy for diabetic peripheral neuropathy (Negi et al., 2010). Sharma, et al. (2009) demonstrated that the combination of resveratrol and 4 amino-1,8-naphthalimide is effective treatment for diabetic peripheral neuropathy.

In summary, this study describes the efficacy of the combination of enalapril, α -lipoic acid and menhaden oil on neural complications of diabetes in a second rodent model of type 2 diabetes. In this study we examined the effect of both an early and late intervention. Even though intervention at the earliest point would likely have the greatest benefit clinically we did find that late intervention was capable of reversing diabetic peripheral neuropathy and in the case of intraepidermal nerve fibers in the skin and corneal nerves in the sub-epithelium and

penetrating the epithelium we found that treatment was able to induce nerve regeneration. Generally we found that combination therapy was more effective against multiple neuropathic endpoints than was mono-therapy. Combination therapy together with good glycemic control and life style modifications such as exercise may be the best option for slowing the progression and reversing diabetic peripheral neuropathy (Kluding et al., 2012; Singleton et al., 2014).

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Figure legends

Figure 1: *Glucose utilization curve for control mice, type 2 diabetic mice and type 2 diabetic mice treated with menhaden oil, α -lipoic acid, enalapril or their combination following early or late intervention.* Data are for mice receiving early intervention (top) or late intervention (bottom). Fasting blood glucose at time 0 for control, type 2 diabetic mice untreated and type 2 diabetic mice treated with menhaden oil, α -lipoic acid, enalapril or their combination for early intervention was 145 ± 15 , $340 \pm 25^*$, $234 \pm 21^{*,+}$, $286 \pm 30^*$, $245 \pm 25^{*,+}$ and $206 \pm 13^+$ mg/dl respectively; for late intervention was 114 ± 7 , $291 \pm 18^*$, $194 \pm 17^{*,+}$, $249 \pm 17^*$, $195 \pm 18^{*,+}$, $159 \pm 6^+$, respectively (* $p < 0.05$, compared to control mice; + $p < 0.05$, compared to untreated diabetic mice). Data are the mean \pm S.E.M. The area under the curve (AUC) was significantly different, $p < 0.01$ (impaired), for type 2 diabetic mice and type 2 diabetic mice treated with menhaden oil, α -lipoic acid or enalapril vs. control (early intervention top). There was a significant improvement, $p < 0.05$, in glucose utilization in type 2 diabetic mice treated with the combination therapy when compared to untreated type 2 diabetic mice or the diabetic mice treated with monotherapies (early intervention top). Glucose utilization by type 2 diabetic mice treated with the combination therapy remained significantly impaired, $p < 0.05$, compared to control mice. The AUC was significantly different, $p < 0.01$ (impaired), for type 2 diabetic mice and type 2 diabetic mice treated with menhaden oil, α -lipoic acid or enalapril alone or the combination of all three vs. control (late intervention bottom). The number of mice in each group was the same as shown in Tables 1 and 3 for early and late intervention, respectively.

Figure 2: *Representative images of corneal nerves (see arrows) in the sub-epithelial layer as observed by corneal confocal microscopy (left), corneal nerves in the sub-epithelial layer as visualized following collection of the cornea, fixation and immunohistochemistry (middle), and*

corneal nerves penetrating the epithelium in the region of the whorl as visualized following collection of the cornea, fixation and immunohistochemistry (right). The corneas analyzed for these images came from a control mouse. Scale bar = 500 μm for middle image and 50 μm for right image.

Figure 3: *Effect of early treatment of type 2 diabetic mice with menhaden oil, α -lipoic acid, enalapril or their combination on innervation of sub-epithelial layer of the cornea as determined by corneal confocal microscopy (left) and anti-tubulin III immunostaining of the cornea sub-epithelial layer (middle) or corneal epithelial layer (right).* The experimental procedures and analyses are described in the Materials and Methods section. The number of mice in each group was the same as described in Table 1. Total duration of diabetes was 16 weeks. Corneal nerve images as shown in Figure 2 were analyzed from all mice following *in vivo* corneal confocal microscopy (left sub-epithelial corneal nerves, expressed as mm/mm^2) or following *in vitro* anti-tubulin III immunostaining of sub-epithelial corneal nerves (middle, expressed as % of corneal surface area) and following *in vitro* labeling of corneal nerves penetrating the epithelium in the region of the whorl by anti-tubulin III immunostaining (right, expressed as corneal nerve volume). Data are presented as the mean \pm S.E.M. * $p < 0.05$ compared to control mice; + $p < 0.05$ compared to diabetic mice by one-way analysis of variance with Bonferroni's test for multiple comparisons.

Figure 4: *Effect of late treatment of type 2 diabetic mice with menhaden oil, α -lipoic acid, enalapril or their combination on innervation of sub-epithelial layer of the cornea as determined by corneal confocal microscopy (left) and anti-tubulin III immunostaining of the cornea sub-epithelial layer (middle) or corneal epithelial layer (right).* Description of this Figure is the same as Figure 3 except that the mice used in this study were in the later treatment study group. The experimental procedures and analyses are described in the Materials and Methods section.

The number of mice in each group was the same as described in Table 3. Total duration of diabetes was 24 weeks. Data are presented as the mean \pm S.E.M. * $p < 0.05$ compared to control mice; + $p < 0.05$ compared to diabetic mice by one-way analysis of variance with Bonferroni's test for multiple comparisons.

Table 1: Effect of Early Intervention of Type 2 Diabetic Mice with Menhaden Oil, α -Lipoic Acid, Enalapril or their Combination on Change in Body Weight, Blood Glucose and Serum Triglycerides, Free Fatty Acids, Cholesterol and Liver Nitrotyrosine and Steatosis

Determination	Control	Diabetic	Diabetic + Menhaden Oil	Diabetic + α - Lipoic Acid	Diabetic + Enalapril	Diabetic + Combination
	(19)	(23)	(15)	(14)	(15)	(12)
Start weight (g)	25.1 \pm 0.5	24.6 \pm 0.6	24.9 \pm 0.5	24.8 \pm 0.4	23.2 \pm 0.6	24.8 \pm 0.4
Final weight (g)	29.6 \pm 1.0	38.6 \pm 1.5 [*]	38.8 \pm 2.0 [*]	36.1 \pm 2.0 [*]	29.0 \pm 0.9 [†]	28.9 \pm 1.2 [†]
Blood glucose (mg/dl)	183 \pm 6	422 \pm 19 [*]	359 \pm 29 [*]	368 \pm 30 [*]	354 \pm 36 [*]	238 \pm 18 [†]
Triglycerides (mg/dl)	108 \pm 10	256 \pm 33 [*]	270 \pm 22 [*]	232 \pm 9 [*]	234 \pm 23 [*]	59 \pm 5 ^{*,†}
Free fatty acids (mmol/l)	0.26 \pm 0.02	0.32 \pm 0.02	0.27 \pm 0.02	0.27 \pm 0.04	0.30 \pm 0.04	0.15 \pm 0.02 ^{*,†}
Cholesterol (mg/ml)	1.1 \pm 0.3	3.2 \pm 0.3 [*]	1.4 \pm 0.2 [†]	0.6 \pm 0.2 [†]	1.0 \pm 0.2 [†]	1.1 \pm 0.2 [†]
Nitrotyrosine (pmol/mg protein)	7.6 \pm 0.3	10.8 \pm 0.5 [*]	14.9 \pm 0.8 ^{*,†}	11.0 \pm 0.5 [*]	11.0 \pm 0.6 [*]	7.2 \pm 0.4 [†]
Steatosis (% area)	4.1 \pm 0.7	45.7 \pm 2.4 [*]	38.7 \pm 4.3 [*]	34.1 \pm 4.3 [*]	28.7 \pm 4.0 ^{*,†}	5.4 \pm 1.1 [†]

Data are presented as the mean \pm S.E.M. * P < 0.05 compared to control mice; † P < 0.05 compared to diabetic mice. Parentheses indicate the number of experimental animals.

Table 2: Effect of Early Intervention of Type 2 Diabetic Mice with Menhaden Oil, α -Lipoic Acid, Enalapril or their Combination on Motor and Sensory Nerve Conduction Velocity, Thermal Nociception and Intraepidermal Nerve

Determination	Control	Diabetic	Fiber Density			
			Diabetic + Menhaden Oil	Diabetic + α - Lipoic Acid	Diabetic + Enalapril	Diabetic + Combination
	(19)	(23)	(15)	(14)	(15)	(12)
MNCV (m/sec)	39.1 \pm 1.6	25.8 \pm 0.7 [*]	39.9 \pm 1.7 [†]	37.3 \pm 2.0 [†]	37.2 \pm 2.0 [†]	39.9 \pm 1.9 [†]
SNCV (m/sec)	28.9 \pm 0.6	22.9 \pm 0.3 [*]	28.8 \pm 0.5 [†]	26.7 \pm 0.7 ^{*,†}	25.6 \pm 0.5 ^{*,†}	28.3 \pm 0.2 [†]
Thermal nociception (sec)	5.3 \pm 0.1	7.8 \pm 0.2 [*]	6.8 \pm 0.1 ^{*,†}	7.0 \pm 0.1 ^{*,†}	7.0 \pm 0.1 ^{*,†}	6.6 \pm 0.2 ^{*,†}
INFD (profiles/mm)	26.0 \pm 0.5	15.5 \pm 0.4 [*]	19.4 \pm 0.4 ^{*,†}	19.2 \pm 0.2 ^{*,†}	18.8 \pm 0.7 ^{*,†}	22.6 \pm 0.6 [†]

Data are presented as the mean \pm S.E.M. * P < 0.05 compared to control mice; † P < 0.05 compared to diabetic mice. Parentheses indicate the number of experimental animals.

Table 3: Effect of Late Intervention of Type 2 Diabetic Mice with Menhaden Oil, α -Lipoic Acid, Enalapril or their Combination on Change in Body Weight, Blood Glucose and Serum Triglycerides, Free Fatty Acids, Cholesterol and Liver Nitrotyrosine and Steatosis

Determination	Baseline Control	Baseline Diabetic	Control	Diabetic	Diabetic + Menhaden Oil	Diabetic + α -Lipoic Acid	Diabetic + Enalapril	Diabetic + Combination
	(19)	(23)	(19)	(23)	(15)	(14)	(15)	(12)
Start weight (g)	25.9 \pm 0.5	25.7 \pm 0.6	24.8 \pm 0.4	25.9 \pm 0.5	25.6 \pm 0.7	25.7 \pm 0.4	25.4 \pm 0.6	25.8 \pm 0.6
Weight at treatment (g)	NA	NA	28.8 \pm 0.4	39.9 \pm 1.4 [*]	41.3 \pm 1.3 [*]	42.1 \pm 1.7 [*]	40.8 \pm 1.3 [*]	41.4 \pm 1.1 [*]
Final weight (g)	30.7 \pm 0.5	41.6 \pm 1.8 [*]	31.8 \pm 0.5	47.9 \pm 3.1 [*]	49.5 \pm 1.9 [*]	43.3 \pm 2.8 [*]	31.6 \pm 1.1 [†]	31.0 \pm 0.7 [†]
Blood glucose at treatment (mg/dl)	NA	NA	176 \pm 5	322 \pm 19 [*]	300 \pm 24 [*]	339 \pm 15 [*]	338 \pm 29 [*]	337 \pm 21 [*]
Blood glucose (mg/dl)	189 \pm 8	407 \pm 35 [*]	186 \pm 8	338 \pm 37 [*]	237 \pm 23	285 \pm 30	313 \pm 42 [*]	252 \pm 13
Triglycerides (mg/dl)	85 \pm 9	126 \pm 19 [*]	93 \pm 10	149 \pm 26 [*]	80 \pm 24 [†]	55 \pm 10 [†]	53 \pm 17 [†]	32 \pm 6 ^{*,†}
Free fatty acids (mmol/l)	0.23 \pm 0.03	0.29 \pm 0.03	0.27 \pm 0.02	0.42 \pm 0.06	0.24 \pm 0.03 [†]	0.29 \pm 0.02	0.24 \pm 0.04 [†]	0.17 \pm 0.02 [†]
Cholesterol (mg/ml)	1.3 \pm 0.1	2.8 \pm 0.1 [*]	1.2 \pm 0.1	2.8 \pm 0.2 [*]	2.3 \pm 0.1 [*]	2.3 \pm 0.2 [*]	2.7 \pm 0.2 [*]	2.3 \pm 0.2 [*]
Nitrotyrosine (pmol/mg protein)	8.7 \pm 0.3	12.0 \pm 0.5 [*]	9.8 \pm 0.4	12.0 \pm 0.6 [*]	14.1 \pm 0.5 [*]	10.9 \pm 0.4	10.0 \pm 0.4	10.6 \pm 0.6
Steatosis (% area)	4.9 \pm 0.5	35.7 \pm 2.1 [*]	3.7 \pm 0.6	53.5 \pm 2.7 [*]	30.8 \pm 2.2 ^{*,†}	30.2 \pm 2.1 ^{*,†}	21.0 \pm 2.2 ^{*,†}	9.6 \pm 0.6 [†]

Data are presented as the mean \pm S.E.M. * P < 0.05 compared to respective control mice; † P < 0.05 compared to diabetic mice. NA = not applicable. Parentheses indicate the number of experimental animals.

Table 4: Effect of Late Intervention of Type 2 Diabetic Mice with Menhaden Oil, α -Lipoic Acid, Enalapril or their Combination on Motor and Sensory Nerve Conduction Velocity, Thermal Nociception and Intraepidermal Nerve Fiber Density

Determination	Baseline Control	Baseline Diabetic	Control	Diabetic	Diabetic + Menhaden Oil	Diabetic + α -Lipoic Acid	Diabetic + Enalapril	Diabetic + Combination
	(8)	(7)	(11)	(10)	(11)	(11)	(12)	(11)
MNCV (m/sec)	39.5 \pm 1.6	26.6 \pm 1.7 [*]	40.5 \pm 1.2	26.2 \pm 0.9 [*]	44.3 \pm 2.3 [†]	34.6 \pm 2.5	35.8 \pm 2.5	44.4 \pm 3.2 [†]
SNCV (m/sec)	30.4 \pm 0.6	23.5 \pm 0.7 [*]	25.9 \pm 1.0	18.7 \pm 0.3 [*]	25.5 \pm 0.6 [†]	22.7 \pm 1.2 [†]	23.3 \pm 0.7 [†]	24.9 \pm 0.5 [†]
Thermal nociception (sec)	5.1 \pm 0.1	8.4 \pm 0.2 [*]	5.2 \pm 0.1	9.0 \pm 0.2 [*]	6.1 \pm 0.1 ^{*,†}	6.8 \pm 0.1 ^{*,†}	6.4 \pm 0.1 ^{*,†}	6.1 \pm 0.2 ^{*,†}
INFD (profiles/mm)	24.7 \pm 0.6	16.1 \pm 0.6 [*]	26.0 \pm 0.7	15.1 \pm 0.4 [*]	22.3 \pm 0.6 ^{*,†}	20.6 \pm 0.5 ^{*,†}	21.9 \pm 0.7 ^{*,†}	22.6 \pm 0.6 ^{*,†}

Data are presented as the mean \pm S.E.M. * P < 0.05 compared to respective control mice; † P < 0.05 compared to diabetic mice.

Parentheses indicate the number of experimental animals.

Figure 1

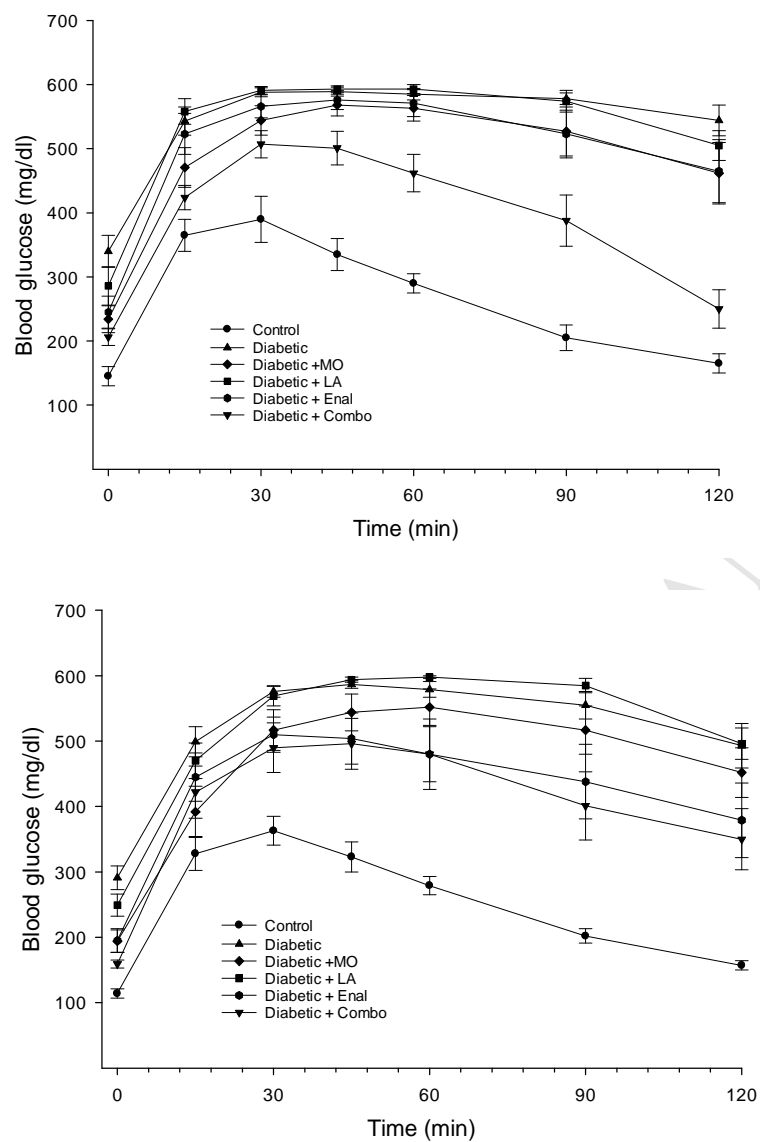
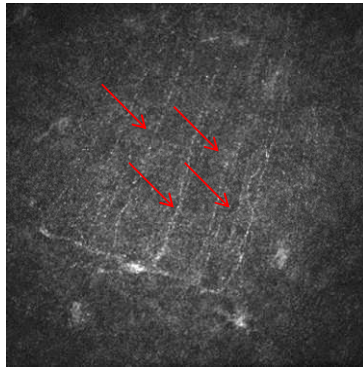
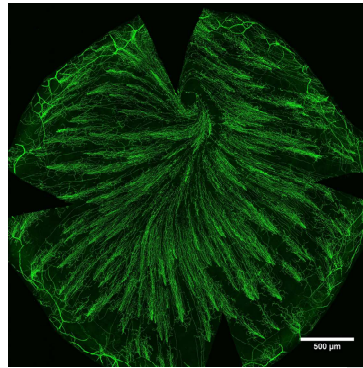


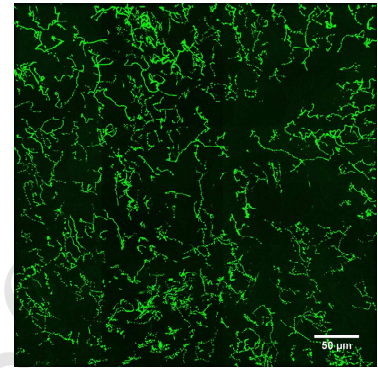
Figure 2



Sub-epithelial corneal
nerves via corneal
confocal microscopy



Immunostaining of
sub-epithelial corneal
nerves



Immunostaining of
corneal nerves in the
epithelial layer

Figure 3

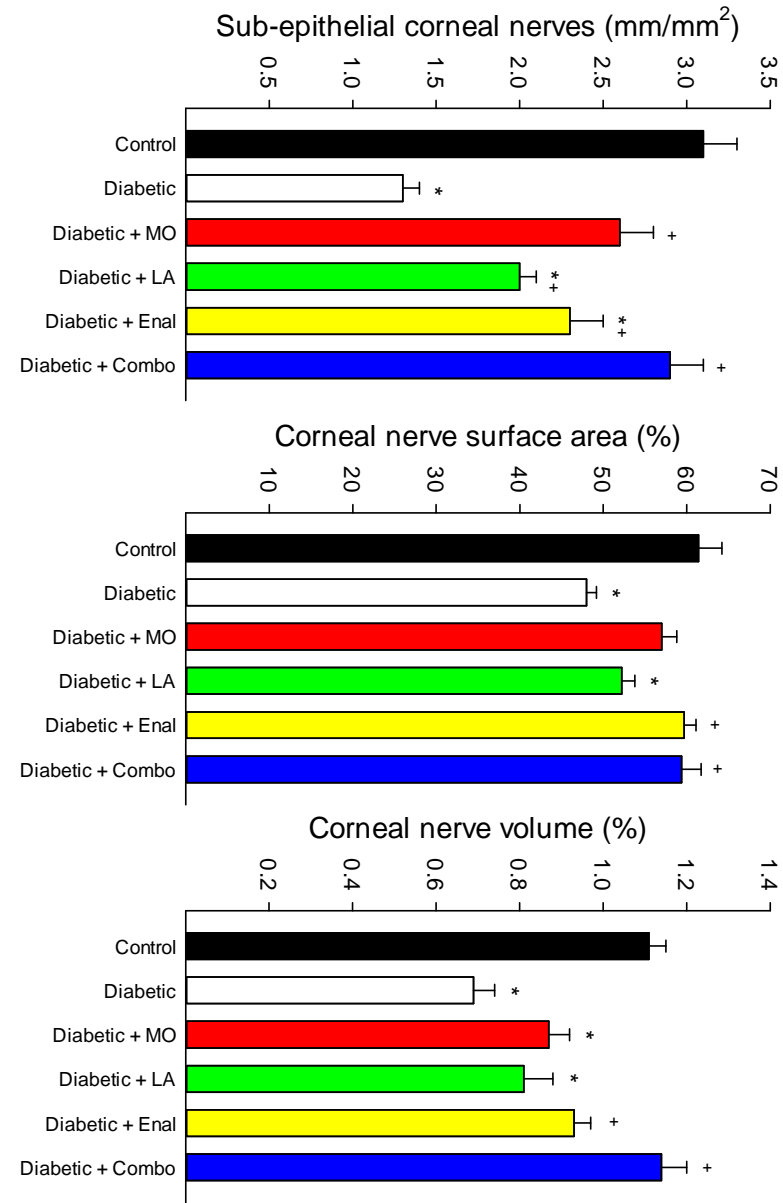


Figure 4

