Combination Therapy To Delay The Progression Of Diabetic Induced Peripheral Neuropathy In Rats.

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Abstract: Diabetes was induced in male Wistar rats by administrating streptozotocin (52mg/kg,i.p). Diabetic rats were treated with combination of gamma linolenic acid (30mg/kg,p.o), alpha lipoic acid (30mg/kg,p.o), phloroglucinol (250mg/kg,p.o) 
I,thyroxine (1mg/kg,s.c), in one group and gamma linolenic acid (30mg/kg,p.o), alpha lipoic acid (30mg/kg,p.o), allantoin (200mg/kg,p.o),I-thyroxine (1mg/kg,s.c), in other group for eight weeks. The degree of protection was determined by measuring motor co-ordination, sensitivity to a mechanical stimulus, thermal hyperalgesia, grip strength, sciatic nerve conduction velocity, glycosylated haemoglobin (GHb), serum nitrate/nitrite levels and anti-oxidant status in sciatic nerve. Combination drugs treatment significantly improved the body weight, behavioral and biochemical parameters in treated diabetic groups when compared to diabetic control rats. Glycemic control was maintained in diabetic rats treated with the combination drugs resulting in the protection against diabetic induced peripheral neuropathy.

Keywords: Diabetic peripheral neuropathy, streptozotocin, gamma linolic acid, alpha lipoic acid, phloroglucinol, allantoin, I-thyroxine.

1. Introduction

Diabetic peripheral neuropathy is a common microvascular complication affecting more than 50% of the diabetic patients(1). People with diabetes can, over time, develop nerve damage throughout the body. Some people with nerve damage have no symptoms. Others may have symptoms such as pain, tingling, or numbness—loss of feeling—in the hands, arms, feet, and legs. Nerve problems can occur in every organ system, including the digestive tract, heart, and sex organs.

Distal symmetric neuropathy is the commonest accounting for 75% DN. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030(2) and the projected rise in the global burden of diabetes is spurring an increase in neuropathy.

The pathogenesis of diabetic peripheral neuropathy (DPN) is complex and resulting from contributions of various pathways. Type 1 and type 2 diabetes will elevate the blood sugar levels followed by initiation of various pathways;

Polyl Pathway
Excess amounts of glucose are present in both type 1 and type 2 diabetes resulting in saturation of hexokinase which leads to entering of excess glucose into polyl pathway where aldose reductase reduces glucose into sorbitol. Since, sorbitol cannot cross cell membranes and hence it accumulates to produce osmotic stresses on cells by drawing water into the insulin-independent tissues. Further the sorbitol dehydrogenase can then oxidize sorbitol to fructose.

Hexosamine biosynthetic pathway
Hyperglycemia mediates hexosamine pathway which produce transforming growth factor(TGF-β1), a prosclerotic cytokine causally involved in the neuropathy.

Advance glycation end(AGEs) product
Glycation is the non-enzymatic reaction between reducing sugars such as glucose and proteins, lipids or nucleic acid. Formation of AGEs is a complicated molecular process involving simple and more complex multistep reactions. AGEs contributes to develop peripheral neuropathy and AGEs formation is encourage by hyperglycemia.

Diacylglycerol pathway
Diacylglycerol is produced by hydrolysis of phospholipid phosphatidylinositol 4,5 – bisphosphate(PIP2), activates protein-serine. threonine kinase belonging to the proein kinaseC family. Many of which play important role in the control of cell growth and differentiation. PKC activates other intracellular targets including a cascade of protein kinase known as mitogen activated protein kinase(MAPK) pathway, leading to transcription factor phosphorylation, change in gene expression and stimulation of cell proliferation. The above pathway will contribute to increased oxidative stress, pro-inflammatory gene

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expression, alters protein functions, endothelial damages, platelet activation which eventually cause peripheral neuropathy.

A number of different agents from diverse chemical classes have entered clinical trials for the treatment of metabolic abnormalities in DPN, but only few approved for clinical use while other drugs either ineffective or withdrawn (2). Current treatment options for symptomatic treatment of DPN include antidepressants, anticonvulsants. These agents are modestly effective for symptomatic relief, but they do not affect the underlying pathology nor do they slow progression of the disease (3). Over the years lots of effort was made to develop one ideal or uniform outcome for patients seeking relief from diabetic peripheral neuropathy using a single drug. Unfortunately, no single drug has proved capable of delaying the progression of diabetic peripheral neuropathy. Hence, in my current project a novel attempt was made to delay the progression of diabetic peripheral neuropathy using combination of different drugs.

Levo-thyroxine is a synthetic thyroid hormone that is chemically identically to thyroxine (T₄) which is naturally secreted by the follicular cells of the thyroid gland. It has been reported that L-thyroxine provides neurotropic support and also has the action of regeneration of myelin sheath. In this experiment racemic form of thyroxine is not used because dextro form is cardiotoxic in nature. Thyroid hormones (TH) [T₄ (tetraiodothyronine) and T₃ (triiodothyronine)], the only iodine containing compounds with biological activity, TH stimulate synthesis of Na+/K+ ATPase and also regulates metabolism by stimulating protein synthesis and increase the use of glucose and fatty acids for ATP production. They also increase lipolysis and enhance cholesterol excretion (4).

Phloroglucinol(1,3,5-trihydroxybenzene) is themonomic building unit phlorotannins, phenolic compound known only brown algae(phaeophyceae). Phloroglucinol has been reported to have inhibitory activity in the formation of advanced glycation endproducts (ADEs) and also provides anti-hyperglycaemia and good anti-oxidant(5,6).

Allantoin (5-ureidohydantoin[2,5-dioxo-4-imidazolidinyl]urea) : A urea hydantoin that is found in urine and plants and is used in dermatological preparations. Allantoin have anti-diabetic effects by modulating antioxidant activities, lipid profile and by promoting release of glucagon like peptide(GLP-1), thereby improving the function of β-cells maintaining normal insulin and glucose level. Allantoin is also found to increase nitric oxide(NO) levels(7).

α-lipoic acid is an organosulfur compound derived from octanoic acid. It is a cofactor essential in mitochondrial metabolism with anti-oxidant and anti-inflammatory activity. Lipoic acid has been shown to be effective in neuropathy pain treatment in patients with sciatica, carpal tunnel syndrome and diabetic neuropathy(8). The mechanisms of action of ALA in experimental diabetic neuropathy include reduction of oxidative stress along with improvement in nerve blood flow, nerve conduction velocity, and several other measures of nerve function(9).

Gamma linolenic acid (GLA, all cis 6, 9, 12-Octadecatrienoic acid, C18:3, n-6), is produced in the body from linoleic acid (all cis 6,9-octadecadienoic acid), an essential fatty acid of omega-6 series by the enzyme delta-6-desaturase. It is found primarily in vegetable oils. The most significant source of GLA for infants is breast milk. GLA is further metabolized to dihomogamma linolenic acid (DGLA) which undergoes oxidative metabolism by cyclooxygenases and lipoxygenases to produce anti-inflammatory eicosanoid. GLA and its metabolites also affect expression of various genes where by regulating the levels of gene products including matrix proteins. These gene products play a significant role in immune functions and also in cell death (apoptosis)(10).

2. Material and method

2.1 Animals

Adult rats of Wistar strain weighing 200-250g were included for the study. Animals were housed in polypropylene cages on clean paddy husk bedding. Animals were kept in an environment with controlled temperature at 25°C±2°C and exposed to 12hr light/dark cycle. All the animals were provided with fresh food and water ad libitum. Wistar rats were administered with single injection of streptozotocin (55 mg/kg. of body weight i.p. in 100 mM sodium citrate buffers, pH 4.5). After 72 hours, blood were collected from the tail vein for the determination of fasting blood glucose. Fasting blood glucose more than 250 mg/dl were considered as diabetic and were used for the preventive studies. Body weight (final body weight) was also measured after 72 hours Animals which did not comply with above criteria, and which were found to be diseased were excluded from the study. After one-week adaptation period,
the healthy animals were used for the study. All the protocols were approved by Institutional Animal Ethical Committee. IAEC NO: AACP/P-48, India.

2.2 Equipments and chemicals
Information about equipments and chemicals are given in table 2.

2.3 Experimental design
Twenty rats were randomly divided into four groups (Group I-Group IV). Group I served as normal control group. Group II, Group III and IV were induced diabetic rats and included in the study as experimental rats. Group II served as diabetic control group whereas Group III received Gamma linolenic acid, Alpha lipoic acid,Phloroglucinol,l-Thyroxine and Group IV received Gamma linolenic acid, Alpha lipoic acid,Allantoin,l-Thyroxine daily for eight weeks. Treatment was started after diabetes was confirmed in rats. Rats were also administered insulin (3IU/day, s.c.) (11) for the complete period of the study.

2.4 Dose selection
The LD50 of l-thyroxine is 50mg/kg(s.c) which means up to 5mg/kg(s.c) of l-thyroxine that is one tenth (1/10) of its LD50 dose can be used for therapeutic proposes. In current study we used 1mg/kg(s.c) to check whether the chosen dose of l-throxine along with other combination drugs have positive effect on diabetic neuropathy or not.

The LD50 of phloroglucinol and allantoin is 4000mg/kg (p.o) and 10g/kg(p.o). The dose of 250mg/kg(p.o) of phloroglucinol and 200mg/kg(p.o) of allantoin was selected because the same dose shown positive effect on diabetic neuropathy in rats [The studies were done in our college].(12)

The LD50 of alpha lipoic acid is 2000mg/kg (p.o).In this study very low dose of alpha lipoic acid (30mg/kg,p.o) was used to associate the effect of low dose alpha lipoic acid on the diabetic peripheral neuropathy in rats.

It has reported that 40mg/kg, p.o of gamma linolenic acid exerts anti-inflammatory and anti-fibrotic effects in diabetic neuropathy(13). 30mg/kg, p.o of gamma linolenic acid was used in present study to check the efficacy of lower dose of gamma linolenic acid along with other combination drugs.

2.5 Assessment of body weight
To assess the general condition of animals, they were examined daily for clinical signs such as alopecia, piloerction or hind limb weakness and mortality. Body weight was measured using digital balance (Essae® DS-252). Loss of body weight was compared between body weight measured at the beginning and at the end of the study.

2.6 Electrophysiological parameters
Measurement of Nerve Conduction Velocity (NCV): The left sciatic nerves were then placed in a moist nerve chamber (MLT016/B AD Instruments, Australia) to measure NCV. NCV was measured by stimulating proximally at the sciatic notch by stimulating electrode (MLA270 AD Instruments, Australia) with 10 mV at 1Hz to 5 Hz and the action potential was measured using recording electrodes (MLA 285) by placing distally to the sciatic notch. NCV was calculated by using the following formula:

Nerve Conduction Velocity(NCV) = m/sec = distance/latency

2.7 Behavioral parameters
2.7.1Assessment of thermal hyperalgesia (14)
Tail immersion test (warm water): Rat’s tail was marked at lower 5 cm portion and immersed in warm water bath (46ºC). The withdrawal response of tail or sign of struggle was observed and the reaction time was recorded using stop watch (cut-off time 12 sec). The tail was dried carefully after recording every response. The reaction time was recorded before and after treatment in normal, diabetic and treated groups. Reductions in tail withdrawn time specify hyperalgesia.

2.7.2Rota-Rod performance test (15): Motor coordination was evaluated by a Rota-Rod apparatus. The Rota-Rod unit consists of a rotating rod, 75mm in diameter, which was divided into four parts by compartmentalization to permit the testing of four rats at a time. Briefly, in a training session, the rats were placed on the rod that was set to 15 RPM and the performance time that each rat was able to remain on the rota-rod was recorded. The rats were subjected to three training trials from 3h-4h intervals on two separate days for acclimatization purposes. In the test session, the rats were placed on the rota-rod and their performance times were recorded. All the readings were taken in triplicate.

2.7.3Grip Strength measurement (16): The grip strength meter was used for evaluating grip
strength of the animals. Before commencement of the experiment the animals were acclimatized by placing them on the instrument for a few minutes. Rats were held by the tail above the grid of grip strength meter to an almost horizontal position. The base of the tail was then pulled following the axle of the sensor until it released the grid. The force achieved by the animal was then displayed on the screen and was recorded as Newton.

2.7.4 Mechanical Hyperalgesia (17):
The nociceptive flexion reflex was quantified using a fabricated Randall-Selitto paw pressure device which applies a linearly increasing mechanical force (g) to the dorsum of the rat’s hind paw. Nociceptive threshold, expressed in grams, was applied by increasing pressure to the hind paw until a squeak (vocalization threshold) was elicited. As this test involves animal handling, the rat was used to being handled as following: 3 days before the experiment, rats were handled without escaping from the hand for 20 s, 2 or 3 times depending on their capacity to be quiet. On the day of the experiment, rats were again handled 2–3 times for 20 s. No rats should show aversive reaction during handling. Then, the paw of the rat was placed under the tip, and the progressive pressure applied until the rat vocalised. The vocalisation threshold was measured 3 or 4 times in order to obtain two consecutive values that differed no more than 10%, and respecting an interval of at least 10 min between two measures.

Table 1
Effect of treatment of combination of drugs for eight weeks on biochemical parameters in diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal rats</th>
<th>Diabetic rats</th>
<th>Diabetic rats+comb.1</th>
<th>Diabetic rats+comb.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (%)</td>
<td>1.667±0.147***</td>
<td>13.43±1.198</td>
<td>10.15±0.3603**</td>
<td>10.08±0.3591**</td>
</tr>
<tr>
<td>Serum NO(µg/ml)</td>
<td>39.15±1.048***</td>
<td>80.92±1.322</td>
<td>74.93±1.048**</td>
<td>76.52±0.9635*</td>
</tr>
<tr>
<td>Malonaldehyde(nmol/mg)</td>
<td>2.658±0.365***</td>
<td>6.58±0.342</td>
<td>3.45±0.1538***</td>
<td>3.25±0.204***</td>
</tr>
<tr>
<td>GSH(µg/mg)</td>
<td>97±22±0.766***</td>
<td>60.54±1.218</td>
<td>85.73±0.756***</td>
<td>81.05±0.621***</td>
</tr>
<tr>
<td>SOD(U/mg)</td>
<td>18.30±0.658***</td>
<td>7.35±0.264</td>
<td>15.87±0.359***</td>
<td>16.55±0.283***</td>
</tr>
<tr>
<td>Catalase(µ/mol/min/mg)</td>
<td>84.13±1.361***</td>
<td>49.97±1.358</td>
<td>71.37±1.236***</td>
<td>73.40±1.106***</td>
</tr>
</tbody>
</table>

HbA1c, glycosylated haemoglobin; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; comb.1, alpha lipoic acid, gamma linolenic acid, phloroglucinol, l-thyroxine; comb.2, alpha lipoic acid, gamma linolenic acid, allantoin, l-thyroxine. Values are represented as mean±SEM(n=5). One Way ANOVA followed by Tukey’s Multiple Comparison Test. *P<0.05, **P<0.01, ***P<0.001 vs diabetic control group.

2.7.5 Mechanical allodynia (18): Rats were placed individually on an elevated mesh (1cm2 perforations) in a clear plastic cage and adapted to the testing environment for at least 15 min. To assess withdrawal threshold of the rats hind paw, the polypropylene tip was used. Tip choice depends on the tissue being probed. Rigid tips work well for the less sensitive, tougher hind paw tissue while semi-flexible tips may be a better choice for a more tender area such as the ano-genital ridge. Rigid tip was mounted on the cone of the probe. Ideally, two investigators were needed to measure hind paw withdrawal threshold (mechanical sensitivity) using an EvF apparatus - one to operate the apparatus and carry out the measurements, and another to transcribe the recorded measurements and handle rats/mice. All measurements were performed in a quiet, temperature-controlled room, and at the same time of day. Positive responses were noted if the paw was robustly and immediately withdrawn. Paw withdrawal threshold was defined as the minimum pressure required to elicit withdrawal reflex of the paw, at least one time on the five trials. Voluntary movement associated with locomotion were not considered as a withdrawal response. Mechanical allodynia was defined as a significant decrease in withdrawal thresholds to electronic von-Frey.

2.8 Biochemical parameters
2.8.1 Blood withdrawal
Tail vein blood sample collection
Procedure
- Wistar rats were kept comfortable in a restrainer while maintaining the temperature around 24 to 27°C.
- The tail is dipped into warm water (40°).
- Local aesthetic cream was applied on the surface of the tail 30 min before the experiment.
- A 23G needle was inserted into the blood vessel and blood was collected using a capillary tube or a syringe with a needle.
- After completed blood collection silver nitrate ointment was applied to stop the bleeding.
- Restrainer was washed frequently to avoid/prevent pheromonally induced stress or cross infection.

Table 2. Equipments and chemicals

<table>
<thead>
<tr>
<th>SL NO</th>
<th>Equipments and instruments</th>
<th>Model/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Analytical balance</td>
<td>Acculab</td>
</tr>
<tr>
<td>2</td>
<td>Cooling microfugeCM-12</td>
<td>Servewellinstruments</td>
</tr>
<tr>
<td>3</td>
<td>Homogenizer</td>
<td>Servewellinstruments</td>
</tr>
<tr>
<td>4</td>
<td>Cyclomixer</td>
<td>Remiequipments</td>
</tr>
<tr>
<td>5</td>
<td>UV-Visible spectrophotometer</td>
<td>Thermo electron – 100</td>
</tr>
<tr>
<td>6</td>
<td>Bacteriological incubator</td>
<td>Servewellinstruments</td>
</tr>
<tr>
<td>7</td>
<td>Actophotometer</td>
<td>Techno</td>
</tr>
<tr>
<td>8</td>
<td>Powerlab</td>
<td>ADinstruments</td>
</tr>
<tr>
<td>9</td>
<td>Nervechamber</td>
<td>ADinstruments</td>
</tr>
<tr>
<td>10</td>
<td>Semi auto analyser</td>
<td>Artos</td>
</tr>
<tr>
<td>11</td>
<td>Electronic vonfrey instrument</td>
<td>Sine automations</td>
</tr>
<tr>
<td>12</td>
<td>Randall-selitto instrument</td>
<td>Sine automations</td>
</tr>
</tbody>
</table>

Table 2.1 Chemicals
All chemicals used were of analytical grade

<table>
<thead>
<tr>
<th>SL NO</th>
<th>Chemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Streptozotocin</td>
<td>Sigma</td>
</tr>
<tr>
<td>2</td>
<td>Gamma linolenic acid</td>
<td>Dr. Reddy’s</td>
</tr>
<tr>
<td>3</td>
<td>Alpha lipoic acid</td>
<td>JIGS chemical</td>
</tr>
<tr>
<td>4</td>
<td>Allantoin</td>
<td>Himedia</td>
</tr>
<tr>
<td>5</td>
<td>Phloroglucinol</td>
<td>Himedia</td>
</tr>
<tr>
<td>6</td>
<td>L-thyroxine</td>
<td>Sigma</td>
</tr>
<tr>
<td>7</td>
<td>5,5 dithiobis 2-nitrobenzoic acid</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>8</td>
<td>Trichloro aceticacid</td>
<td>S.D fine. chem. Ltd</td>
</tr>
<tr>
<td>9</td>
<td>Thiobarbituric acid</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>10</td>
<td>EDTA</td>
<td>Rankem</td>
</tr>
<tr>
<td>11</td>
<td>Hydrogen peroxide</td>
<td>S.D fine. chem. Ltd</td>
</tr>
<tr>
<td>12</td>
<td>Di-Potassium hydrogen orthophosphate</td>
<td>Titan biotech</td>
</tr>
<tr>
<td>13</td>
<td>N-(1-naphyl)-ethylene diaminediochloride</td>
<td>S.D fine. chem. Ltd</td>
</tr>
<tr>
<td>14</td>
<td>Ortho phosphoric acid</td>
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</tr>
<tr>
<td>15</td>
<td>Hanilamide</td>
<td>S.D fine. chem. Ltd</td>
</tr>
<tr>
<td>16</td>
<td>Sodium carbonate</td>
<td>S.D fine. chem. Ltd</td>
</tr>
<tr>
<td>17</td>
<td>TrisHCl</td>
<td>Titan biotech</td>
</tr>
<tr>
<td>18</td>
<td>Sodium nitrate</td>
<td>Reachem laboratory chemicals</td>
</tr>
<tr>
<td>19</td>
<td>Sodium hydroxide</td>
<td>Merck</td>
</tr>
<tr>
<td>20</td>
<td>Potassium sodium tartrate tetrahydrate</td>
<td>Merck</td>
</tr>
<tr>
<td>21</td>
<td>Sodium dihydrogen orthophosphate</td>
<td>Thomas Baker chemicals</td>
</tr>
<tr>
<td>22</td>
<td>Copper sulphate</td>
<td>Rankem</td>
</tr>
</tbody>
</table>
2.8.2 Glycosylated hemoglobin (GHb) (19): At the end of study 8 weeks blood was withdrawn from tail vein of rat and collected in EDTA tubes. The GHb was determined by using commercially available kit. A hemolysed preparation of whole blood is mixed continuously for 5 minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the hemolysate preparation. During the mixing, HbAo binds to the ion exchange resin leaving GHb free in the supernatant. After the mixing period, a filter separator is used to remove the resin from the supernatant. The percent GHb is determined by measuring absorbance of the GHb fraction and the total hemoglobin (THb) fraction.

2.8.3 Isolation of sciatic nerve
The rats were anesthetized by intraperitoneal administration of thiopentone sodium, 30 mg/kg [11]. After anesthesia, rat backs were shaved and NCV was recorded. Briefly incision is made at L4-L6 spinal segments. The sciatic nerves were surgically exposed from sciatic notch to the L6 spinal segments. The sciatic nerves were rapidly removed carefully impregnated on fine filter paper to remove any accompanying blood, soaked for 10 minutes in Ringer-Locke buffer to prevent spontaneous firing of the nerve (16).

2.8.4 Preparation of nerve homogenate: A segment of sciatic nerve, approximately 1.5 cm in length, 5 mm proximal and 5 mm distal was used for preparing the 10% w/v homogenates for biochemical estimation. Tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 1000 rpm 4°C for 3 min and the supernatant divided into two portions, one of which was used for measurement of lipid peroxidation (LPO) and the remaining supernatant was again centrifuged at 12,000 rpm at 4°C for 15 min and used for the measurement of endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT) glutathione (GSH) (21). Measurement of lipid peroxidation: The extent of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured according to the method of Esterbauer and Cheeseman. Tissue extracts were mixed separately with 1 ml TCA (20%), 2 ml TBA (0.67%) and heated for 1 h at 100°C. After cooling, the precipitate was removed by centrifugation. The absorbance of each sample was measured at 535 nm using a blank containing all the reagents except the sample. As 99% TBARS are malondialdehyde (MDA), so TBARS concentrations of the samples were calculated using the extinction coefficient of MDA, which is 1.56×105 M-1 cm-1 and were expressed as µM of malondialdehyde per mg protein (22). Measurement of superoxide dismutase (SOD) and catalase activity: Sciatic nerve homogenate was centrifuged at 4°C, 17,500 xg for 10 min. Supernatant was used for the measurement of SOD activity by pyrogallol autooxidation method (5) and catalase activity by H2O2 degradation method, which is a quantitative spectroscopic method developed for following the breakdown of H2O2 at 240 nm in unit time. The sample readings were taken by placing 1 ml of phosphate buffer and 100 µl of tissue homogenate in the reference cuvette and 1 ml of H2O2 and 100 µl of homogenate in the test cuvette in the spectrophotometer. For each measurement, the reading was taken at 240 nm 1 min after placing the cuvettes in Shimadzu spectrophotometer (23).

2.8.5 Measurement of nitric oxide concentration in sciatic nerve homogenate.
The assay mixture consist of medium (100 µL) and Griess reagent (100 µL) place in round-bottom 96-well tissue culture plates (incubation time 30 min) and absorption will measure at 570 nm on a UV-Visible spectrophotometer. The amount of nitrite will determine by comparison of unknowns with a NaNO2 standard curve. The nitrite detection limit is 0.20 nM.

Lipid peroxidation(superoxide dismutase(4) superoxide dismutase (SOD),catalase activity(4)), and reduced glutathione(GSH) (4) were done according to published papers.

2.9 Statistical analysis
Statistical evaluations were done by ANOVA, expressed as mean ± S.E.M. followed by Tukey’s multiple comparison test using Graph Pad Prism 5 computer program. P<0.05 was considered statistically significant.

3. Results
3.1 Assessment of body weight
The percentage change in body weight of normal and diabetic rats at 8th week was found to be 17.72±1.66g and -24.53±2.706g. The body weight
of diabetic rats was significantly reduced (P<0.001) as compared to normal control, similarly the change of body weight of diabetic treated with comb.1 and comb.2 was found to be -12.49±0.93g and -12.30±2.457g which significantly improved (P<0.001) as compared to diabetic control rats (Figure 1).

### 3.2 Measurement of non invasive sciatic nerve conduction velocity (NCV)

NCV was significantly (P<0.001) reduced in eight weeks diabetic control rats (27.20 ± 0.534secc) when compared to normal control rats (48.11 ±0.6sec). The diabetic rats treated with comb.1 and comb.2 shows significantly (P<0.001) improved in NCV (37.22 ±0.9192secs, 35.76±0.9058 secs ) (Figure 2).

### 3.3 Behavioral studies

#### 3.3.1 Thermal hyperalgesia:

The tail flick latencies in hot immersion test of normal and diabetic control rats was found to be 4.8±0.386secs, 9.0±0.836secs respectively and the tail flick latencies of diabetic rats was significantly increased(P<0.001) as compared to normal control rats. Tail flick latencies of diabetic rats treated with comb.1 and comb.2 was found to be 6.64±0.1806secs, 5.380±0.2818secs and same was significantly (P<0.001) improved when compared to diabetic control rats. (Figures 3).

#### 3.3.2 Measurement of motor coordination using rota rod:

Fall of time at 15 rpm of normal and diabetic rats was found to be 235.3±1.753 and 37.58±2.343secs respectively, and the latency of diabetic rats was significantly reduced (P<0.001) as compared to normal control. Latency in diabetic rats treated with comb.1 and comb.2 was found to be 89.65±1.088secs and 70.24±0.7346secs and same was significantly (P<0.001) improved when compared to diabetic control rats. (Figure 4).

#### 3.3.3 Measurement of grip strength:

The grip strength of normal and diabetic rats was found to be 12.45±0.1205N and 5.660±0.5250N respectively, the grip strength of diabetic rats was significantly reduced (P<0.001) as compared to normal control. The grip strength of diabetic rats treated with comb.1 and comb.2 was found to be 8.96±0.6112N and 8.660±0.25N and same was significantly improved P<0.001 as compared to diabetic control rats. (Figure 5).

#### 3.3.4 Mechanical hyperalgesia:

Mechanical threshold for normal and diabetic control rats was found to be 241.8±2.577g and 127.2±0.8602g respectively and the mechanical threshold of diabetic rats was significantly lower(P<0.001) when compared to normal control rats. Mechanical threshold of diabetic rats treated with comb.1 and comb.2 was found to be 216.6±1.077g and 208.6±1.536g and same was significantly higher(P<0.001) when compared with diabetic control rats.(Figure 6).

#### 3.3.5 Mechanical allodynia:

Paw withdrawal threshold for normal and diabetic control rats was found to be 7.620±0.317g,5.180±0.1772g respectively and the paw withdrawal threshold of normal rats was significantly higher(P<0.001) when compared to diabetic rats. Paw withdrawal threshold of diabetic rats treated with comb.1 and comb.2 was found to be 6.220±0.037g,5.940±0.0509g and same was significantly higher(P<0.01,P<0.05) when compared with diabetic control rats.(Figure 7).

### 3.4 Biochemical studies

%Glycosylated haemoglobin,serum nitric oxide and malonaldehyde levels were significantly increased while the levels of glutathione, superoxide dismutase, catalase were significantly reduced in diabetic rats when compared to normal rats. Treatment with comb.1 and comb.2 both statistically improved the biochemical parameters when compared to diabetic control rat.(Table 1).

### 4. Discussion

The present study has demonstrated the neuroprotective effect of administration of comb.1 containing gamma linolenic acid, alpha lipoic acid, phloroglucinol,l-thyroxine and Comb.2 containing gamma linolenic acid, alpha lipoic acid, allantoin,l-thyroxine to alleviate the persistence of neuropathic pain in diabetic rats.

Gamma linolenic acid is metabolized to produce anti-inflammatory agent. This anti-inflammatory agent inhibits inflammatory cell infiltration by abrogating increases in monocyte chemotactrant protein-1(MCP-1) and intercellular adhesion molecule-1(ICAM-1) expression and also ameliorate extracellular matrix accumulation. Alpha lipoic acid and allantoin have an antioxidant which helps turn glucose into energy.Alpha lipoic acid and allantoin also attack free radical waste product created when the body turns food into energy. Its ability to kill free radicals may help to treat diabetic peripheral neuropathy. Furthermore, allantoin increased insulin secretion levels due to
the increased function of pancreatic β-cells. L-thyroxine provided neurotropic support and also has the action of regeneration of myelin sheath. It also increased the use of glucose and fatty acids for ATP production. In this experiment racemic form of throxine is not used because dextro form is cardiotoxic in nature. Phloroglucinol has been reported to have inhibitory activity in the formation of advanced glycation endproducts (ADEs) and also provided anti-hyperglycemia and good anti-oxidant activity (5,6).

Hence, the combination drugs used in the current project shown anti-inflammatory, anti-oxidant, improved the function of β-cells and also inhibited the formation of ADEs which may be the reasons for the improved behavioral and biochemical parameters in diabetic rats treated with combination therapy.

We found a significant reduction in the body weight of the diabetic rats as compare to the normal control group. Reduction in the body weight in type 1 diabetes can be due to oxidative stress and altered protein functions which resulted from hyperglycemia. Improved body weight in diabetic groups treated with combination drugs may be due to decrease in oxidative stress and improved general health conditions. According to the previous studies, rats frequently focus on their reaction to the nociceptive stimuli because of the lack of quantifiable signs of spontaneous pain. The nociceptive tests used in the study are thermal hyperalgesia, mechanical hyperalgesia and mechanical allodynia. These tests are used to measure the latency or withdrawal threshold of the rats when exposed to various stimulation. Primarily hyperalgesia is mainly due to sensitization of nociceptive nerve ending and thermal hyperalgesia involved sensitization of unmyelinated nociceptors. A marked decrease in nociceptive threshold in STZ induced diabetic rats as compared to normal control rats indicates the development of significant hyperalgesia. In STZ induced diabetic neuropathy causing hyperalgesia is often accompanied by motor in coordination and reduced muscle strength. Significant reduction in falling latency on rota rod apparatus in diabetic rats indicates impaired motor function and coordination due diabetic neuropathy whereas in the treated groups the rota rod performance was improved when compared to diabetic control groups.

In type 1 and type 2 diabetes mellitus there will be increase in glycosylation of hemoglobin due to hyperglycemia resulting in formation of glycosylated hemoglobin (GHB). Increased %GHB is one of the main reason for the development of peripheral neuropathy. In current study it was found that the % GHB of treated groups are lowered when compared to diabetic rats. Hence, the combination drugs reduce the rate of glycosylation of hemoglobin by lowering the blood glucose levels.

Significantly improved sciatic nerve conduction velocity in the treated diabetic rats may be due to reduction of sorbitol formation and lowering of oxidative stress which improves the flow of electrical impulse down the stream. Improved flow of electrical impulse is associated with improve physiologic conditions.

Nitric oxide (NO) is a ubiquitous free radical that sets as a neurotransmitter, neuromodulator and intracellular second messenger. Nitric oxide levels were significantly lowered in treated diabetic rats then diabetic control rats. Hence, indicates that combination therapy prevents cellular damage due to nitric oxide.

Prolong hyperglycemia leads to oxidation of lipids which will generate free radicals. These free radicals will cause harmful chemical reactions that can damage cells. The combination treatment shown reduction in lipid peroxidation status when compared with diabetic control rats. The levels of anti-oxidants such as glutathione, catalase and superoxide dismutase was found to be increased in comb.1 and comb.2 treated groups. Antioxidants is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides and heavy metals.

No related side effects was observed in experimental rats and also there was no increased in side effects in experimental rats when the combination of drugs was used to treat diabetic peripheral neuropathy in diabetic rats. Hence, the combination therapy used in the current study was found be safe.

Conclusion:

As such there is no significant difference (according to Tukey’s Multiple Comparision Test) between comb.1 and comb.2 drugs therapy used in current project. However, comb.1 which is gamma linolenic acid, alpha lipoic, L-thyroxine and phloroglucinol shown more improved results or more closer results to normal control rats when compared with comb.2. Hence, comb.1 will be more preference to delay the progression of diabetic peripheral neuropathy when compared with comb.2.
The authors would like to conclude that the supplementation of above combination drugs delays the diabetic induced peripheral neuropathy in rats and we are hoping that the same combination of drugs will show effective results in clinical trial in near future.

Author contribution
My co-author has helped me in each step of this study and also in writing this article.

Figure 1: Effect of treatment of combination of drugs for eight weeks on % body weight change in diabetic rats. Values are represented as mean ± SEM (n=5). One Way ANOVA followed by Tukey’s Multiple Comparison Test. ***P<0.001, **P<0.01 Vs diabetic control group.

Figure 2: Effect of treatment of combination of drugs for eight weeks on nerve conduction velocity in diabetic rats. Values are represented as mean ± SEM (n=5). One Way ANOVA followed by Tukey’s Multiple Comparison Test. ***P<0.001 Vs diabetic control group. Comb.1, gamma linolenic acid, alpha lipoic acid, phloroglucinol, l-thyroxine; Comb.2, gamma linolenic acid, alpha lipoic acid, allantoin, l-thyroxine.

Figure 3: Effect of treatment of combination of drugs for eight weeks on tail flick latencies(46°C) in diabetic rats. Values are represented as mean ± SEM (n=5). One Way ANOVA followed by Tukey’s Multiple Comparison Test. ***P<0.001, *P<0.05 Vs diabetic control group. Comb.1, gamma linolenic acid, alpha lipoic acid, phloroglucinol, l-thyroxine; Comb.2, gamma linolenic acid, alpha lipoic acid, allantoin, l-thyroxine.

Figure 4: Effect of treatment of combination of drugs for eight weeks on muscle incordination by rota rod performance in diabetic rats. Values are represented as mean ± SEM (n=5). One Way ANOVA followed by Tukey’s Multiple Comparison Test. ***P<0.001 Vs diabetic control group.
Comb.1, gamma linolenic acid, alpha lipoic acid, phloroglucinol, l-thyroxine; Comb.2, gamma linolenic acid, alpha lipoic acid, allantoin, l-thyroxine.

Figure 5: Effect of treatment of combination of drugs for eight weeks on grip strength in diabetic rats. Values are represented as mean ± SEM (n=5). One Way ANOVA followed by Tukey’s Multiple Comparison Test. ***P<0.001 Vs diabetic control group.

Comb.1, gamma linolenic acid, alpha lipoic acid, phloroglucinol, l-thyroxine; Comb.2, gamma linolenic acid, alpha lipoic acid, allantoin, l-thyroxine.

Figure 6: Effect of treatment of combination of drugs for eight weeks on mechanical threshold in diabetic rats. Values are represented as mean ± SEM (n=5). One Way ANOVA followed by Tukey’s Multiple Comparison Test. ***P<0.001 Vs diabetic control group.

Comb.1, gamma linolenic acid, alpha lipoic acid, phloroglucinol, l-thyroxine; Comb.2, gamma linolenic acid, alpha lipoic acid, allantoin, l-thyroxine.

Reference


