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ORIGINAL ARTICLE / ÖZGÜN ARAŞTIRMA

The effectiveness of evening primrose oil and alpha lipoic acid in recovery of nerve function in diabetic rats

Diyabetik ratların sinir işlevlerinin düzelmesinde gecesafası yağı ve alfa lipoik asidin etkinliği

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ABSTRACT

Objectives: Diabetic polyneuropathy is a serious complication of diabetes mellitus and the most frequent neuropathy worldwide. Evening primrose oil (EPO) is rich in omega-6 essential fatty acid component and gamma-linolenic acid. Alpha lipoic acid (ALPA) has a protective effect against lipid peroxidation and helps in scavenging free radicals. Data regarding the effect of treatment with EPO on diabetic parameters and neuropathic manifestations are conflicting. This study aimed to determine the therapeutic efficacy of EPO and ALPA in correcting diabetic parameters and functional and structural neuropathic manifestations in streptozotocin (STZ) induced diabetic rats.

Materials and methods: In this study, the effects of two week oral treatment with EPO (1.25 g/kg) was compared to that of ALPA (100 mg/kg) and insulin (2 IU/day), utilized singly or in combination.

Results: Compared with untreated diabetic rats, EPO and ALPA resulted in reduction of serum levels of glucose ($p<0.05$), total cholesterol ($p<0.01$), triglycerides ($p<0.01$), low density lipoprotein cholesterol ($p<0.01$), thiobarbituric acid reactive substance (a marker of oxidative stress) ($p<0.05$), and increased in levels of high density lipoprotein cholesterol ($p<0.05$) and total antioxidant capacity ($p<0.05$). Enhanced positive effect was observed with combination therapy.

Conclusion: This work indicates that EPO and ALPA, particularly when used in combination, improve glycemic control, lipid abnormalities and antioxidant capacity, thus restore the impaired functional properties of peripheral nerves to a great extent. *J Clin Exp Invest 2011; 2 (3): 245-253.*

Key words: Diabetic peripheral neuropathy, evening primrose oil, oxidative stress, alpha lipoic acid

ÖZET

Amaç: Diyabetik polinöropati diabetin en ciddi bir komplikasyonudur ve dünyada en yaygın olan nöropatidir. Akşamsefası yağı (EPO) omega-6 esansiyel yağ asit ve gamalinoleik asit bakımından zengindir. Alfa lipoik asit (ALPA) lipid peroksidasyonuna karşı koruyucu bir etkiye sahiptir ve serbest oksijen radikallerini temizlemeye yardımcı olur. EPO tedavisinin diyabet parametreleri ve nöropatik belirtileri üzerine etkisi konusundaki veriler çelişkilidir. Bu çalışma EPO ve ALPA'nın, streptozotosinle diyabet geliştirilen ratlarda, diyabetik parametreleri ve fonksiyonel ve yapısal nöropatik belirtileri düzeltmedeki tedavi etkinliğini belirlemeyi amaçlamıştır.

Gereç ve yöntem: Bu çalışmada EPO ile iki haftalık oral tedavi (1.25 g/kg) ALPA (100 mg/kg) ve insülin (2 U/gün) ile tek başına ve kombinasyon şeklinde karşılaştırıldı.

Bulgular: Tedavi edilmemiş ratlarla karşılaştırıldığında; EPO ve ALPA, serum glukoz ($p<0.05$), total kolesterol ($p<0.01$), trigliserid ($p<0.01$), düşük yoğunluklu lipoprotein kolesterol ($p<0.01$) ve bir oksidatif stres belirteci olan tiobarbitürik asit reaktif maddesi düzeyinde azalma) ve yüksek yoğunluklu lipoprotein kolesterol ($p<0.05$ ve total antioksidan kapasitede ($p<0.05$ artışa neden oldu. Kombinasyon tedavisi ile artmış pozitif etki gözlendi.

Sonuç: Bu çalışma EPO ve ALPA'nın özelikle birlikte kullanıldığında glisemik kontrol, lipid bozuklukları ve antioksidan kapasiteyi düzelterek, bozulmuş periferik sinir fonksiyonlarını büyük ölçüde iyileştireceğini düşündürmektedir. *Klin Deney Ar Derg 2011; 2 (3): 245-253.*

Anahtar kelimeler: Diabetik periferik nöropati, akşamsefası yağı, oksidatif stres, alfa lipoik asit

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INTRODUCTION

Diabetes mellitus (DM) is often associated with neuropathic, cardiovascular, retinal and renal complications. Evidence from several studies indicated that the prevalence rates of diabetic complications are proportionately related to the magnitude and duration of antecedent hyperglycemia.[1] Diabetic neuropathy (DN) is a common complication of DM. Results from animal and human studies indicate that DN is a highly dynamic disorder; both degeneration and regeneration are present simultaneously. However, over time, the balance between degeneration and regeneration shifts toward more degeneration.[2] Schwann cells and myelin forming cells of peripheral nerves are important in the regenerative process. These cells are impaired in DM as a result of hyperglycemia, hypoxia, oxidative stress[3] and loss of regenerative capacity of nerve fibers.[4] Several potential mechanisms are suggested as underlying causes of diabetic microangiopathy and neuropathy, which include: a) abnormal fatty acid metabolism (particularly the lower ratio of desaturation of linoleic acid to gamma-linolenic acid)[5], b) abnormal intracellular metabolism as changes in peripheral nerve polyol metabolism, c) changes in Na^+-K^+- exchanging ATPase activity, d) depletion of myo-inositol[6], e) disruption of prostaglandin to thromboxane A2 ratio in favor of vasoconstriction together with increase in platelet aggregation[7], f) reduction of nerve blood flow and hypoxia[8], g) impairment of cellular scavenging activity against oxidative stress[9], h) decrease in specific isoforms of protein kinase (PKC)[10], and i) loss of growth factor systems.[11] As a result, number of agents have been experimentally investigated and showed some ability to prevent or reverse diabetes-induced changes. These agents include: myo-inositol[12], aldose, reductase inhibitors[13], aminoguanidine,[14] nerve growth factor,[15,16] antioxidants[17-20] and nutrients rich in polyphenolic antioxidants (such as green tea, garlic, olive oil and ginger).[21]

Evening primrose oil (EPO) is one of the most widely used herbal medicines in various parts of the world. EPO is extracted from the seeds of Oenothera biennis. It is found in fields and roadsides in the United States and Canada.[22,23] EPO has attracted attention as a medicinal plant as the oil of its seeds contains high concentrations of gamma-linoleic acid (GLA). EPO demonstrates therapeutic efficacy in some conditions like eczema, asthma, rheumatoid arthritis, breast problems and metabolic disorders.[24-27] EPO is a rich source of the omega-6 essential fatty acids such as GLA (a precursor of prostaglandin E1) and linoleic acid (LA). GLA and LA are essential components of myelin and neuronal cell membrane. In DM, the first step in the conversion of LA to GLA is disturbed leading to shortage of GLA and its metabolites as prostaglandins (PGs) and prostacyclin.[27-29]

Alpha lipoic acid (ALPA) has a protective effect against lipid peroxidation.[30] ALPA may have a direct scavenging activity on free radicals. It may enhance the activity of other natural antioxidants and antioxidant enzymes in tissues and peripheral nerves such as reduced glutathione, vitamin C and vitamin E, catalase and superoxide dismutase (SOD).[31-36]

Data regarding the effects of EPO on glycemic control, lipid profile and manifestations of peripheral neuropathy are controversial and poorly understood.[37-40] Here, we evaluated the effects of EPO, ALPA and insulin (used singly or in combination) on some diabetic parameters [as blood glucose level, lipid profile {total cholesterol (TC), triglycerides (TG), low (LDL) and high density (HDL) lipoprotein cholesterol}, nitrate (an end product of nitric oxide oxidation), thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, and total antioxidant capacity (TAOC), an indicator of antioxidant reserve] and electrophysiological manifestations associated with DN.

MATERIALS AND METHODS

Experimental design

This study was conducted on adult male streptozotocin (STZ) induced rats. Their weight ranged between 200-250 grams. The experiment was conducted in accordance with international standards on animal welfare as well as being compliant with local and national regulations of practice for the care and use of animals for scientific purposes. Adequate measures were taken to minimize pain or discomfort. The protocol of this study was approved by the institutional animal experimentation ethics committee of Assiut University, Assiut, Egypt.

Rats were divided into the following groups; each consisting of 10 rats: 1) Group 1: non-diabetic

control rats, 2) Group 2: non-diabetic rats treated orally with EPO, 3) Group 3: untreated STZ induced diabetic rats, 4) Group 4: STZ induced diabetic rats treated orally with EPO, 5) Group 5: STZ induced diabetic rats treated orally with ALPA, 6) Group 6: STZ induced diabetic rats treated orally with EPO and ALPA in similar doses as groups 4 and 5, 7) Group 7: STZ induced diabetic rats treated with subcutaneous (s.c) insulin, and 8) Group 8: STZ induced diabetic rats treated with EPO and insulin.

Diabetes was induced by single intraperitoneal (i.p.) injection of STZ (Sigma chemical Co., USA) in a dose of 65 mg/kg and freshly dissolved in sterile 0.9% saline solution. The non-diabetic control rats were injected with the same volume of sterile 0.9% saline solution. Blood glucose concentration was determined 72 hours after STZ injection. The diabetic rats included in the study had blood glucose concentrations >13.89 mmol/L. Diabetic rats were left untreated for 6 weeks. This period is enough for the development of diabetic complications.[41] The tested substances were then given to the rats by gavage for 2 weeks. EPO (Pharco for Pharmaceuticals & Medical Application Company, Egypt) was given orally in a dose of 1.25 g/kg daily. ALPA (EVA Pharm for Pharmaceuticals & Medical Application Company, Egypt) was given orally in a dose of 100 mg/kg daily.[33] Insulin (Mixtard 30HM, Biphasic Isophane Insulin Injection 30/70) (Biosynthetic Human Insulin, Novo Nordisk, Denmark) was given subcutaneously (s.c.) in a dose of 2 IU daily.[42]

From all groups of rats, blood collection was done twice: after 6 weeks of induction of DM and after 2 weeks treatment with the tested substances (or 8 weeks after induction of DM). Blood sampling was done after a period of fasting for determination of the following biochemical parameters: 1) The blood glucose level was determined using enzymatic colorimetric method kit supplied by Roche diagnostics, (GmbH, D-68298 Mannheim, USA)[43]; 2) The serum lipid profile (TC, TG, LDL and HDL) was determined using enzymatic colorimetric kits supplied by Roche diagnostics, (GmbH, D-68298 Mannheim, USA).[44-46] LDL was calculated using the difference between TC and HDL.[46] For glucose and lipids, the absorbance of the standard substance and blank was read at 500 nm using a spectrophotometer (Pharmacic LKB Ultraspec Plus, England, Cambridge), 3) Nitrate (one of the stable and non volatile end-products of nitric oxide or NO oxidation) was determined using Griess reagent (Biochem for laboratory fine chemicals, USA)[47], 4) Thiobarbituric acid reactive substance (TBARS) was determined colorimetrically (MP Biomedicals, Inc., France),[48] and 5) TAOC was determined (MP Biomedicals, Inc., France) spectrophotometrically (Pharmacic LKB Ultraspec Plus, England, Cambridge).[49] Blood samples were kept at -20oC until the time of analysis.

Clinical and electrophysiological manifestations of DN were also assessed twice: after 6 weeks of induction of DM and after 2 weeks treatment with the tested substances (or 8 weeks after induction of DM) as follow: a) thermal and mechanical pain was determined using hot plate test (Ugo basile biological research apparatus, Model 24780, Comerio-Va-Italy) by measuring the latency to thermal threshold for each rat[50], b) the nociceptive mechanical threshold was determined using the Paw pressure test (biological research apparatus, Model 24880, Comerio-Va-Italy)[51], c) motor coordination was determined using the rotarod test [52] (Treadmill for Rats 7700, Ugo basile biological research apparatus, Comerio-Va-Itally) , and d) Nerve conduction velocity (NCV) was determined for the sciatic nerve using an invasive procedure in a temperature controlled environment[29] as follow:

Rats were anesthetized with an i.p. injection of urethane dissolved in saline in a dose of 1-1.5 g/kg. The sciatic nerve in the right leg was exposed at three sites. The two proximal (stimulating) sites were located near the sciatic notch (1st) and at ~15 mm distal (2nd), and the distal (recording) or 3rd site was located at the distal end of the tibial nerve above the foot. The stimulating and recording electrodes were placed directly under the sciatic nerve. The nerve was bathed in paraffin and maintained at 37°C under radiant heat. The sciatic nerve was stimulated by monophasic voltage pulses delivered directly to the nerve at 20% above threshold for the myelinated A-fibers. The evoked potentials were recorded using Harvard Universal Oscillograph (serial No.400, England). Action potentials from both stimulating sites were averaged six to eight times. Following NCV recordings, sutures were placed around the sciatic nerve at the sites of the stimulating electrodes. NCV was calculated by dividing the distance of nerve segment between the two stimulat-

ing sites measured in millimeters by the difference between proximal and distal latencies measured in milliseconds.

Statistical Analysis

Statistical Science for Social Package (SPSS Inc, USA) software computer program version 10 was used for data analysis. Data were presented as mean ± S.E.M (standard error of mean). One-way ANOVA was used to provide information on whether there is a main effect of treatment. LSD was used to determine whether there are differences between specific treatment groups. LSD comparisons were corrected for multiple comparisons. For all tests, a probability (P) < 0.05 was considered significant.

RESULTS

STZ induced diabetic rats demonstrated increase in blood glucose level (>13.89 mmol/L) at 72 hours compared to that of non-diabetic control rats. Glucose level remained higher ($p<0.001$) till the end of the experiment. STZ induced diabetic rats demonstrated increase in serum levels of TC ($p<0.001$), TG ($p<0.01$), LDL-c ($p<0.01$) and TBARS ($p<0.001$) and decrease in serum levels of HDL-c ($p<0.001$), nitrate ($p<0.001$) and TAOC ($p<0.001$). Treatment of non-diabetic rats with EPO did not result in changes in serum levels of glucose, TC, TG, LDL, HDL, nitrate, TBARS and TAOC (Figures 1 and 2).

Compared to untreated STZ induced diabetic rats, diabetic rats treated with EPO, ALPA and insulin demonstrated decrease in the serum levels of blood glucose ($p<0.05$, $p<0.05$, $p<0.001$ respectively), TC ($p<0.01$ for all), TG ($p<0.01$ for all), LDL-c ($p<0.05$ for all) and TBARS ($p<0.05$ for all), and increase in the levels of HDL-c ($p<0.01$, $p<0.01$, $p<0.05$ respectively), nitrate ($p<0.05$ for all) and TAOC ($p<0.05$ for all) (Figures 1 and 2).

Compared to non-diabetic control rats, the untreated diabetic rats demonstrated manifestations of peripheral neuropathy in the form of decease in hot plate latency ($p<0.001$), mechanical pain threshold ($p<0.001$), the time spent on rotarod ($p<0.01$) and decease in NCV of sciatic nerve ($p<0.01$). Diabetic rats treated with EPO, ALPA and insulin showed a significant increase in hot plate latency ($p<0.05$ for all), mechanical pain threshold ($p<0.05$ for all), the time spent on rotarod ($p<0.05$ for all) and NCV of sciatic nerve ($p<0.05$ for all) (Figure 1 and Table 1).

Comparing combination treatment with EPO and ALPA or EPO and insulin to single treatment with either ALPA or insulin, combination treatment resulted in more decrease in the serum levels of blood glucose ($p<0.05$ for both), TC ($p<0.01$ for both), TG ($p<0.01$ for both), LDL-c ($p<0.01$ for both) and TBARS ($p<0.01$ for both), and more increase in the levels of nitrate ($p<0.05$ for both) and TAOC ($p<0.05$ for both), more improvement in hot plate latency ($p<0.01$ for both) and more increase in mechanical pain threshold ($p<0.01$ for both) (Figure 1 and 2, Table 1).

Table 1. Effect of different treatments on clinical and electrophysiological parameters

	Group	Hot plate latency (S)	Mechanical pain threshold (gm)	Time spent in rotarods (S)	NCV (m/s)
1	Control non-diabetic rats	10.6 ± 0.96	128.9 ± 6.2	121.20 ± 3.73	53.64 ± 2.35
2	Non-diabetic rats treated with EPO	11.33 ± 0.81	129.5 ± 4.5	122.00 ± 3.64	55.13 ± 2.35
3	STZ-diabetic rats	6.25 ± 0.59°°°	89.5 ± 7.3°°°	85.4 ± 4.91°°	41.40 ± 2.16°°
4	STZ-diabetic rats treated with EPO	9.13 ± 0.66*	111.9 ± 6.3*	107.40 ± 5.68*	49.96 ± 2.15*
5	STZ-diabetic rats treated with ALPA	9.27 ± 0.81*	113.5 ± 4.7*	107.90 ± 5.50*	50.06 ± 2.60*
6	STZ-diabetic rats treated with EPO and ALPA	10.43 ± 0.74**	150.4 ± 4.7***,‡‡	109.20 ± 5.54*	51.74 ± 1.76**
7	STZ-diabetic rats treated with insulin	8.82 ± 0.55*	110.7 ± 5.1*	105.70 ± 6.96*	51.25 ± 1.65*
8	STZ-diabetic rats treated with EPO and insulin	9.73 ± 0.65**	147.1 ± 4.4***,‡‡	107.80 ± 5.7*	49.67 ± 1.94**

Data are expressed as mean ± S.E.M

Group 3: STZ-diabetic rats (6 weeks after induction of DM); **Groups 4-8**: STZ-diabetic rats (after 2 weeks treatment) STZ, Streptozotocin; EPO, primrose oil; ALPA, alpha lipoic acid; NCV, nerve conduction velocity; S: second; m/s: meter/second

°°P<0.01, °°°P<0.001 compared to non diabetic control rat; *P<0.05, **P<0.01, ***P<0.001 compared to STZ-diabetic control rats; ‡P<0.05, ‡‡P<0.01, compared to STZ-diabetic rats treated with either EPO or ALPA alone

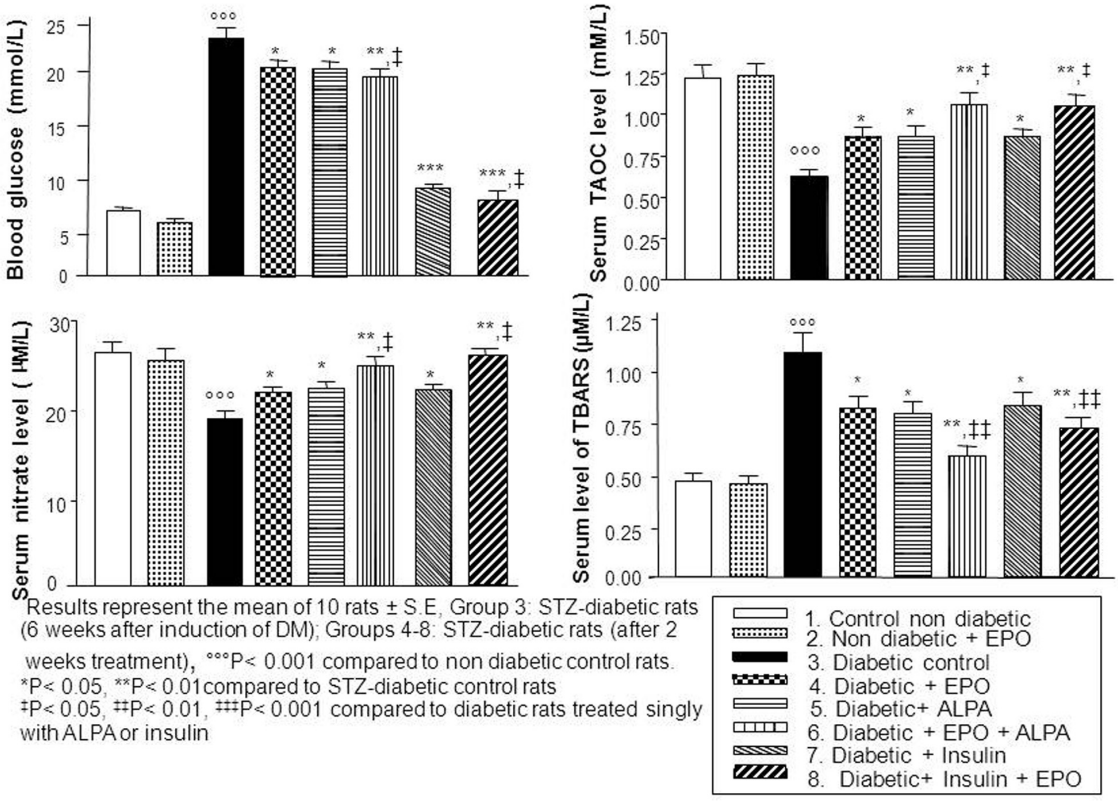

Figure 1. Effect of different treatments on serum biochemistry

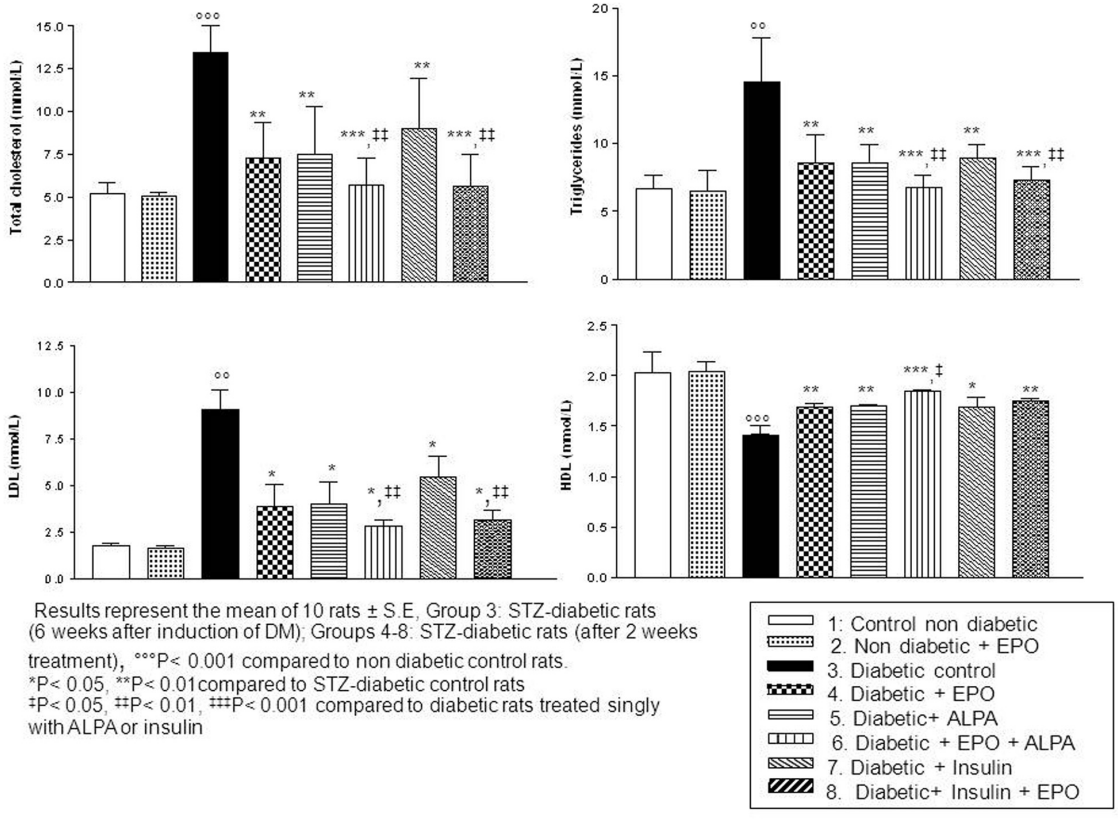

Figure 2. Effect of different treatments on lipid profile

DISCUSSION

The results of this study confirmed that EPO, ALPA and insulin were able to reverse neuropathic manifestations towards normal levels which might be due to: 1) correction of hyperglycemia [17,53], 2) correction of lipid profile[54,55], 3) induction of vasodilatation in tissues[55,56], and 4) antioxidant or free radical scavenging activity.[57]

STZ induces damage of pancreatic cells resulting in hyperglycemia, hypoxia and oxidative stress. STZ increases glycation of collagen and plasma proteins. STZ enhances auto-oxidative reactions of sugars and aggravate damage to both lipids and proteins in the circulation resulting in hypercholesterolemia or dyslipidemia.[54,55] However, some studies reported that in STZ induced diabetic rats, EPO was able to restore NCV of peripheral nerves to its normal levels without reduction of hyperglycemia suggesting a neuroprotective effect.[37,38] In contrast, others reported that EPO was unable to reverse various lipid abnormalities induced by DM[58,59] or even it could increase the levels of TC and TG and reduce the levels of HDL.[54] The conflicting results of different studies could be explained by the differences in materials and methods, age of the tested animals, duration of drug treatments and their prescribed doses.

This study and others showed that EPO, ALPA or insulin were able to increase the levels of nitrate impaired by STZ.[60] Nitrate is an end product of nitric oxide (NO) oxidation.[55] At low physiological levels, NO modulates vascular tone in a similar manner to endothelial-dependent relaxing factor. In addition, NO has a potent free radical scavenging activity.[61] Under physiological conditions, nitric oxide synthase (NOS), a rate forming enzyme of NO, is present in pancreatic β-cells and is involved in the release of insulin.[62] In DM, disturbance in levels of NO and NOS have been suggested to impair endothelium-dependent vasodilatation of pancreatic vascular beds and insulin production resulting in oxidative stress and hyperglycemia.[63]

In experimental models of DM, the increase in levels of lipid peroxides, free radicals and oxidative stress and the decrease in levels of antioxidant reserve, cause oxidation of polyunsaturated fatty acids (PUFAs) which are embedded in the cell membrane.[64] This causes damage of pancreatic cells, hyperglycemia and DM.[65,66] We and others showed that EPO, ALPA and insulin are able to reduce lipid peroxidation and enhance the total antioxidant status of the body as evidenced by reduction in levels of TBARS[67] and increase in levels of TAOC observed in this study as compared to pre-treatment levels.[54,68] TBARS is formed during non-enzymatic auto-oxidation of PUFAs. It is an indicator of lipid peroxidation and oxidative stress burden.[69] TAOC measures the collective activities of wide range of antioxidants such as vitamin E, vitamin C, vitamin A, uric acid, glutathione and antioxidant enzymes including glutathione reductase (GSH.R), glutathione peroxidase (GSH.Px) and superoxide dismutase (SOD).[69]

The results of this study indicate that combination treatment with EPO and ALPA or EPO and insulin is more effective than singular treatment in improvement of biochemical parameters associated with DM and more improvement of manifestations of peripheral neuropathy induced by DM. In general, this therapeutic effectiveness could be due to additive effect, synergistic effect or both. Additive means an augmented net effect through action on the same pathway, while synergism means an augmented net effect through action on two different pathways.

In conclusion, EPO and ALPA are effective in lowering lipid and hemostatic risk factors in STZ induced DN. The therapeutic effects of EPO seemed similar to ALPA and augmented by combined administration with ALPA or insulin. However, whether the beneficiary results of combination treatment on the levels of blood glucose, lipids, nitrate, oxidative stress and antioxidant markers are due to additive or synergistic effect or both, are still unknown. Continuing research efforts are needed to explore the exact mechanisms of EPO in DN.

Conflicts of interest:

None

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