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Biochemical Effect of Alpha-lipoic Acid and Insulin Alone and in Combination on Changes in the Phospholipid Composition in Experimental Diabetic Neuropathy

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ABSTRACT

Diabetic neuropathy is the most common complication of diabetes. We investigate the effect of alpha-lipoic acid and insulin alone and in combination on changes in the phospholipids composition in the sciatic nerve of experimental diabetic neuropathy. A total of 120 male rats were used in this study. The experimental induction of diabetes in rats was induced by a single intraperetinoel (i.p) injection of 50 mg kg^{-1} of streptozotocin (STZ) freshly dissolved in citrate buffer, pH 4.5. After eight weeks of diabetes induction all rats were divided into six main equal groups, 20 animals each. Group I (control group): Received no drugs, Group II (diabetic group), Group III (normal "-lipoic acid-treated group), Group IV (diabetic alpha-lipoic acid -treated group), Group V (diabetic insulin- treated group), Group VI (diabetic alpha-lipoic acid and insulin-treated group). Eight weeks after diabetes induction therapeutic treatment with alpha-lipoic acid (54 mg kg^{-1} b.wt. i.p daily) and insulin (2U s.c daily) were given either alone or in combination and continued for six weeks. Equivalent volumes of saline were given subcutaneously to the rats in the other diabetic and non diabetic control groups. Blood samples and sciatic nerve tissues were collected at 4 and 6 weeks from the onset of treatment for determination of serum glucose and total cholesterol, total phospholipids and membrane phospholipids composition of sciatic nerve. The obtained results revealed that, diabetic neuropathy in rats resulted in marked increase in serum glucose level, sciatic nerve total cholesterol and phosphatidylglycerol contents. Treatment with "-lipoic acid significantly decreased serum glucose, phosphatidylglycerol and sphingomyelin contents with increase in total cholesterol content in sciatic nerve. Insulin treatment significantly increased total phospholipids and markedly decrease phospholipids composition of rat sciatic nerve including phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol and sphingomyelin contents. Meanwhile, treatment with "-lipoic acid and insulin combination significantly decreased total phospholipids concentration and phospholipids composition in rat sciatic nerve including phosphatidylcholine, phosphatidylglycerol and sphingomyelin contents. These results indicate that, alterations in the amounts of phospholipids composition in sciatic nerve could be related to the physiological changes of early diabetic neuropathy. These result suggest that, administration of "-lipoic acid combined with insulin prevent hyperglycemia-induced changes in phospholipids composition suggesting its therapeutic potential in complications of diabetes and dibetes neuropathy.

Key words: Diabetic neuropathy, sciatic nerve, phospholipids composition, alpha-lipoic acid

INTRODUCTION

Diabetes is a group of metabolic changes characterized by elevated blood glucose resulting from defects in insulin secretion, action or both. Chronic high blood sugar of diabetes is associated with long-term damage, dysfunction and eventually a hardware failure, especially the eyes, kidneys, nerves, heart and blood vessels (Huang *et al*., 2005). Diabetes usually associated with increased production of reactive oxygen species and free radicals or antioxidant defenses affected adopted more important to the development and progression of diabetic complications (Kumar *et al*., 2006).

There are two types of diabetes: Type 1 diabetes (T1D) is due to self-destruction of the insulin producing beta cells in the pancreas and type 2 diabetes (T2D) is caused by defects in insulin action and insulin production (Cohen and Horton, 2007). Diabetic neuropathy is the most common complication of diabetes has long resulting in clinically significant medical conditions such as pain, leg ulcers and amputation (Said, 2007). It is believed that a large number of neural mechanisms of anatomical, physiological and neurological neurochimichals contribute to the development and maintenance of diabetic neuropathic pain (Edwards *et al*., 2008; Negi *et al*., 2010) reported that, pathophysiological factors of diabetic neuropathy include persistent hyperglycemia, microvascular insufficiency, oxidative stress, nitrosative stress, PARP over activation, defective neurotrophism, autoimmune-mediated nerve destruction, etc. The factor, such as oxidative stress, AGE and lipid peroxidation formation which produces from the diabetic state stimulates the inflammatory process (Laura *et al*., 2006; Jurevics and Morell, 1994) demonstrated that, almost all of the cholesterol accumulating in sciatic nerve is synthesized *in situ*, indicating that circulating cholesterol is not a significative source of this lipid destined for the myelin membrane. The biosynthesis and accumulation of cholesterol in the sciatic nerve is developmentally regulated and correlates with myelin deposition (Jurevics and Morell, 1994). The mammalian central nervous system contains a large proportion of the PUFAs and significant changes in brain lipid composition occur with aging and in conditions such as diabetes and neurological disorders. Mohanty *et al*. (2000) and Cunha *et al*. (2008) observed that, the lipid content of nerves most likely dominated by myelin lipids and oxidative damage to myelin can credibly promote nerve disorders in diabetic polyneuropathy. Kamboj *et al*. (2009) reported that, on the contrary, phospholipid and ganglioside levels were decreased. Hyperglycemia-induced higher cholesterol to phospholipid ratio reflects the decrease in membrane fluidity.

Lipoic improve antioxidant capacity, taking into account the oxidative stress-induced insulin resistance in type 2 diabetes. Also, Lipoic acid appears to have a potent therapeutic role in addition to its role in management of diabetic neuropathy in protection of diabetic complications due to oxidative stress (El-Nabarawy *et al*., 2010). Therefore, the present study was conducted to investigate the possible protective effects of exogenous administrations of "-lipoic acid, insulin and their combinations on the glycemic control, alterations in total cholesterol, total phospholipids and membrane phospholipids composition of sciatic nerve in streptozotocin- induced diabetic neuropathy in rats.

MATERIALS AND METHODS

Experimental animals: One hundred and twenty white male albino rats, 12-16 weeks old and average body weight 220-250 g were used in this study. Rats were obtained from Laboratory Animals Research Center, Faculty of Veterinary Medicine, Moshtohor, Benha University. Animals

were housed in separated metal cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The animals were fed on constant ration and water was supplied ad-libitum. All animals were acclimatized for minimum period of two weeks prior to the beginning of study.

Drugs used: I-Alpha-lipoic acid (Thiotacid)^{R}: Thiotacid was obtained as pack of five ampoules of 10 mL solution. Each ampoule contains thioctic acid (alpha lipoic acid) 300 mg. Alpha-lipoic acid (Thioctic acid)$^{®}$ manufactured by EVA pharma for pharmaceuticals and Medical Apliances, Egypt. II- Human Insulin(Humulin^{R} U-100): Humulin R presented as regular insulin injection, USP, (recombinant DNA origin) isophane suspension. It consists of zinc-insulin crystals dissolved in a clear fluid. Humulin R manufactured by LILLY Egypt, under License from ELI LILLY U.S.A.

Diabetes induction: Rats were fasted for 18 h and allowed free access of water. The experimental induction of diabetes in male rats was induced by a single intraperetinoel (i.p) injection of 50 mg kg^{-1} of streptozotocin (STZ) freshly dissolved in citrate buffer, pH 4.5. A week later, STZ-treated rats were fasted for 12 h and blood samples were collected from the orbital venous sinus for glucose determination. Only those rats in diabetic group with blood glucose levels higher than 250 mg dL^{-1} were considered diabetic (Ramanathan *et al*., 1999). Diabetic neuropathy in rats were develop within 8 weeks after induction of diabetes (Kumar *et al*., 2005).

Eight weeks after diabetes induction therapeutic treatment with alpha-lipoic acid (54 m kg^{-1} b.wt. i.p daily) and insulin (2 U s.c daily) were given either alone or in combination and continued for six weeks. Equivalent volumes of saline were given subcutaneously to the rats in the other diabetic and non diabetic control groups.

Experimental design: After eight weeks of diabetes induction all rats were divided into six main equal groups, 20 animals each, placed in individual cages and classified as follow: Group I (normal control group): Received no drugs, served as control for all experimental groups. Group II (diabetic non treated group): Received equivalent volumes of saline were given subcutaneously and served as STZ-induced diabetic group. Group III (normal alpha-lipoic acid-treated group): Received "-lipoic acid at a dose level of (54 mg kg^{-1} b.wt. i.p daily) for six week (Gruzman *et al*., 2004). Group IV (diabetic alpha-Lipoic acid-treated group): Received alpha-lipoic acid at a dose level of (54 mg kg^{-1} b.wt. i.p daily) for six week. Group V (diabetic insulin-treated group): Received subcutaneous injection of insulin at a dose level of 2 U each morning for six week (Izbeki *et al*., 2008). Group VI (diabetic alpha-lipoic acid and insulin-treated group): Received alpha-lipoic acid at a dose level of (54 mg kg^{-1} b.wt. i.p. daily) and insulin at dose of 2 U injected subcutaneously each morning for six week.

Sampling: Random blood samples and sciatic nerve specimence were collected from all animal groups (control and experimental group). Two times at 4 and 6 weeks, from the onset of treatment, after eight weeks of diabetes induction.

Blood samples: Blood samples for serum separation were collected after over night fasting by ocular vein puncture at the end of each experimental period and serum was separated by centrifugation at 2500 r.p.m for 15 min. The clean, clear serum was proceed directly for glucose determination.

Sciatic nerve samples: Sciatic nerves from the spin to the peroneal bifurcation were dissected, rinsed in ice-cold saline solution and frozen in liquid nitrogen after removal of adherent tissue. Samples were kept at -80°C in liquid nitrogen until use.

Lipid extraction in sciatic nerve were performed according to the method described by Peuchant *et al*. (1989). On the day of the homogenate preparation sciatic nerve segments were measured, weighed and cut into small pieces and then homogenized with bout 5 mL of isopropanol added to each tube and the tubes were shaken vigorously. After that sufficient amount of anhydrous sodium sulfate was then added to each tube to remove the water. The mixture was vortexed for 2 min and then filtered or centrifuged at 3000 r.p.m. for 10 min. After centrifugation, aliquots were analyzed for the determination of total cholesterol, total phospholipids concentrations and the phospholipids composition. Biochemical analysis: Seurm glucose, in addition to sciatic nerve total cholesterol, total phospholipids concentration were analyzed colorimetrically according to the methods described by Trinder (1969), Allain *et al*. (1974) and Zilversmit and Davis (1950) respectively. Phospholipids composition in sciatic nerve were performed according to the method of Hisham *et al*. (2010).

Statistical analysis: The obtained data were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple test. All analyses were performed using the statistical package for social science (SPSS 13.0 software, 2009). Values of $p<0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Diabetes usually associated with increased production of reactive oxygen species and free radicals or antioxidant defenses affected adopted more important to the development and progression of diabetic complications (Kumar *et al*., 2006). Chronic diabetes with high blood sugar levels is associated with long-term damage, dysfunction and eventually a hardware failure, especially the eyes, kidneys, nerves and cardiovascular system (Vinik and Vinik, 2003). In addition to high blood sugar levels and many other factors involved, such as dyslipidemia, or too fat in the development of cardiovascular complications of diabetes which are the main causes of morbidity and mortality (Reasner, 2008). In patients with diabetes revealed abnormal antioxidant status, auto-oxidation of glucose and glycated proteins excess (Nawale *et al*., 2006; Nishikawa and Araki, 2007).

The recorded data demonstrated in (Table 1) showed significant increase in serum glucose concentration in streptozotocin-induced diabetic neuropathy in rats when compared with the normal control group. These results are nearly similar to those reported by Sayyed *et al*. (2006) who reported that, STZ-induced diabetic rats showed approximately five-fold increase in the blood glucose levels after STZ administration. Dias *et al*. (2005) reported the increase in plasma glucose concentration in diabetic rats. The developed hyperglycemia have been attributed to the specific toxic effects of STZ uptake through glucose transporter-2 (GLUT-2), these toxic effects lead to end organ damage through activation of the aldose reductasc pathway leading to toxic accumulation of sorbitol in nervous system (Greene *et al*., 1988) increased diacyl glycerol synthesis with consequent activation of protein kinase C isoform (PKC) in vascular tissue, initiating diabetic complications (Craven *et al*., 1995) and increased oxidative stress with subsequent alterations in celluar redox balance (Williamson *et al*., 1993). Treatment with "-lipoic acid, insulin and their combination significantly decrease serum glucose leveh in diabetic neuropathy induced in rats allover the experimeal periods. These results are nearly similar to those recorded by Vessal *et al*. (2003) who reported that, oral administration of "-lipoic acid has shown hypoglycemic effects

Table 1: Effects of treatment with alpha-lipoic acid, insulin and their combination on serum glucose, total cholesterol and total phospholipids of sciatic nerve in streptozotocin-induced diabetic neuropathy in male rats

	Glucose (mg dL^{-1})		Total cholesterol (mg g^{-1} tissue)		Total phospholipids (mg g^{-1} tissue)	
Parameter	Four weeks (ẍ±SE)	Six weeks (ẍ±SE)	For weeks (ẍ±SE)	Six weeks (ẍ±SE)	For weeks (ẍ±SE)	Six weeks (ẍ±SE)
Animals groups						
Normal control	117.5±11.09^{d}	123.25±3.54^{d}	18.21±2.83^{c}	9.45±1.03^{b}	35.53±8.00^{c,b}	41.60±1.84^{b}
Diabetic control	582.7 5± 6.75^{a}	446.50±3.88^{a}	21.87±1.52^{c}	39.79±1.65^{a}	48.38±1.56^{a,b}	46.85±1.68^{b}
Normal+"-lipoic acid	131.50±5.58^{c,d}	132.50±2.33^{d}	50.92±0.56^{a}	39.79±4.03^{a}	47.90±3.84^{a,b}	53.99±0.47^{a}
Diabetic+"-lipoic acid	156.50±11.86^{c}	126.00±3.49^{d}	38.20±1.54^{b}	35.49±0.44^{a}	48.15±3.64^{a,b}	42.74±0.96^{b}
Diabetic+insulin	142.50±8.21^{c,d}	153.50±23.45^{b}	36.39±0.50^{b}	6.97±0.34^{b}	52.25±2.60^{a}	55.41±0.75
Diabetic+"-lipoic acid+insulin	192.00±20.51$_{b}$	180.75±16.48^{c}	21.83±0.38^{c}	35.29±11.36^{a}	22.70±6.32^{c}	36.04±3.62^{c}

Data are represented as ẍ± SE,ẍ: Mean values SE: Standard error, Mean values with different superscript letters in the same column are significantly different at p#0.05

against STZ-induced diabetes in rats. These effects can be attributed to the antioxidant supplements that reduce the concentration of glucose in the blood and strengthen the restoration of pancreatic islets and thereby increase the production of insulin in diabetic rats. The blood glucose level was significantly lower than that of untreated diabetics, though all of the insulin-treated diabetic rats were still hyperglycemic (Izbeki *et al*., 2008). In addition, treatment with insulin prevent blood sugar increases and decreases in brain and body weight. These observations are consistent with the overall improvement of insulin treatment of diabetes complications (Nathan *et al*., 2009). Streptozotocin-injected mice had significantly higher blood glucose level. Insulin alone corrected the hyperglycemia and partially reversed the neuropathic pain in diabetic rats (Kuhad and Chopra, 2009). The mechanism that taking supplements "-lipoic acid practicing vascular morphology, possibly via its antioxidant properties as well as carbohydrate and lipid metabolic effects. The current study, supplementation with "-lipoic acid in diabetic rats effectively removes hyperlipidemiat and improves the condition of high blood sugar concentration (Balkis *et al*., 2008). Also, "-lipoic acid reduces free radicals generated during the process of peroxidation and protects the cell structure against damage (Packer *et al*., 2001).

The recorded data showed significant increased of total cholesterol concentration in sciatic nerve of streptozotocin-induced diabetic neuropathy in rats (Table 1). Cholesterol accounts for 20-30% of the total lipids in the Peripheral Nervous System (PNS), in mouse and rabbit sciatic nerves, cholesterol accumulates continuously through out the period of neo-myelinogenesis and during the subsequent period of myelin maturation (Juguelin *et al*., 1986). Normal physiological functioning of the neuronal membrane is highly dependent on its structure and while many factors can influence the membrane fluidity index, the major one is the membrane lipid composition: Cholesterol reduces the membrane fluidity and PUFAs increase it (Yehuda *et al*., 2002). Jurevics and Morell (1994) demonstrated that, almost all of the cholesterol accumulating in sciatic nerve is synthesized *in situ*, indicating that circulating cholesterol is not a significative source of this lipid destined for the myelin membrane. The biosynthesis and accumulation of cholesterol in the sciatic nerve is developmentally regulated and correlates with myelin deposition. Also, Cunha *et al*. (2008) showed that, the lipid content of nerve is likely dominated by myelin lipids and oxidative damage to myelin may plausibly contribute to nerve disorders in diabetic polyneuropathy. Moreover, Kamboj *et al*. (2009) reported that, on the contrary phospholipid and ganglioside levels were decreased. Hyperglycemia-induced increase in cholesterol to phospholipid ratio reflected decrease

in membrane fluidity. The value of total cholesterol concentration of sciatic nerve after four weeks treatment with "-lipoic acid and insulin alone to diabetic rats group significantly increase total cholesterol concentration in sciatic nerve. Meanwhile, six weeks treatment with insulin cause significant decrease of total cholesterol concentration in sciatic nerve compared with diabetic non-treated group. Similarly, Patel and Katyare (2006) showed that, in early diabetic stage of rats the Total Phospholipid (TPL) content of the microsomal membranes of rat brain decreased by19% while the cholesterol (CHL) content increased by 50%. Insulin treatment restored the TPL content whereas the CHL content increased further. A similar 21% decrease in the TPL content persisted at the late stage of diabetes. By contrast, the CHL content increased substantially by 2.4-fold. Insulin treatment had no effect on TPL content but marginally lowered the CHL content. Who added that, in 1 week diabetic animals the TPL and CHL contents of the mitochondria decreased by 34 and 19%, respectively. Insulin treatment partially restored the TPL content but had no effect on CHL content. In 1 month diabetic animals the TPL and CHL contents were unchanged and insulin treatment resulted in lowering the TPL and CHL contents by 15 and 36%. Changes in the gross parameters such as total phospholipid (TPL) and cholesterol (CHL) content in whole brain, synaptic membranes and mitochondria as affected by diabetic condition have been reported. It has also been demonstrated that the diabetic state affects the metabolism of glycolipids except for gangliosides and that the fatty acid composition of the phospholipid classes changes significantly (Kumar and Menon, 1993; Pari and Venkateswaran, 2004). Membrane phospholipids represent a potential influence on the enzymatic properties of the Na^+, K^+-ATPase (Dines *et al*., 1995).

Total phospholipids and phospholipids composition of sciatic nerve: The recorded data in (Table 1) revealed that, a non significant increase in phospholipids concentration in sciatic nerve was observed in streptozotocin induced-diabetic neuropathy in rats. High lipid content in retina and brain is important for the high vulnerability of these tissues to oxidative stress. This is because peroxidative damage of membrane lipids leads to many damages in a cell such as decreases in membrane fluidity, elevated sensitivity to oxidant stress and changes in enzyme activities (Liu and Mori, 1999). Therefore, the important consequences of chronic stress could be attributed to stress-induced lipid peroxidation. Moreover, the increased lipid peroxidation following chronic hyperglycemia was accompanied by a significant increase in the total lipids which can be attributed to increase in the levels of cholesterol, triglycerides and glycolipids (Akpinar *et al*., 2008). Additionaly, Peroxidation of membrane phospholipids has been suspected to be a major mechanism of oxidant injury leading to membrane dysfunction and subsequently to alterations in cellular functions. Cunha *et al*. (2008) showed that, the lipid content of nerve is likely dominated by myelin lipids and oxidative damage to myelin may plausibly contribute to nerve disorders in diabetic polyneuropathy. Changes in the gross parameters such as total phospholipid (TPL) and cholesterol (CHL) content in whole brain, synaptic membranes and mitochondria as affected by diabetic condition have been reported. It has also been demonstrated that the diabetic state affects the metabolism of glycolipids except for gangliosides and that the fatty acid composition of the phospholipid classes changes significantly (Pari and Venkateswaran, 2004). Kamboj *et al*. (2009) reported that, on the contrary phospholipid and ganglioside levels were decreased. Hyperglycemia-induced increase in cholesterol to phospholipid ratio reflected decrease in membrane fluidity. Peripheral diabetic neuropathy is one of the most devastating complications of diabetes mellitus. The pathogenesis of peripheral diabetic neuropathy involves hyperglycemia-initiated mechanisms as well as other factors, i.e., impaired insulin signaling, hypertension, disturbances of fatty acid and lipid metabolism. Membrane phospholipids represent a potential influence on the enzymatic properties of the Na^+, K^+-ATPase (Dines *et al*., 1995).

Treatment with insulin to streptozotocin-induced diabetic neuropathy in rats significantly increased phospholipids concentration of sciatic nerve after six weeks of administration. However, treatment with "-lipoic acid combined with insulin significantly decreased phospholipids concentration allover the periods of the experiments. Currently, little is known about the role of insulin and diabetes in the turnover of membrane phospholipids. A rapid turnover rate is an important feature of membrane phospholipids. The significance of this feature is not well understood and it may result from membrane repair mechanisms and structural changes that may render the membrane more sensitive to activation (Mato and Alemany, 1983). Thus it is reasonable to suspect that tissue function and its pathology will be affected by phospholipids synthesis and hydrolysis. Patel and Katyare (2006) showed that, in early diabetic stage of rats the total phospholipid (TPL) content of the microsomal membranes of rat brain decreased by19% while the cholesterol (CHL) content increased by 50%. Insulin treatment restored the TPL content whereas the CHL content increased further. A similar 21% decrease in the TPL content persisted at the late stage of diabetes. By contrast, the CHL content increased substantially by 2.4-fold. Insulin treatment had no effect on TPL content but marginally lowered the CHL content. Who added that, in 1 week diabetic animals the TPL and CHL contents of the mitochondria decreased by 34 and 19%, respectively. Insulin treatment partially restored the TPL content but had no effect on CHL content. In 1 month diabetic animals the TPL and CHL contents were unchanged and insulin treatment resulted in lowering the TPL and CHL contents by 15 and 36%. The mechanism by which "-lipoic acid supplementation exerts the vascular morphology is probably through its antioxidant properties and the effects on carbohydrate and lipid metabolism. In the current study supplementation with "-lipoic acid in diabetic rats effectively inhibits dyslipidemia and improves the hyperglycemia condition (Balkis *et al*., 2008). Also, "-lipoic acid reduces free radicals generated during the process of peroxidation and protects the cell structure against damage (Packer *et al*., 2001).

The obtained results demonstrated in (Table 2) revealed that, a non significant increase in phosphatidylethanolamine, phosphatidylserine and sphingomyelin and significant increased in

Table 2: Effects of four and six weeks treatment with alpha-lipoic acid, insulin and their combination on phospholipids composition percentage and main phospholipids classes of sciatic nerve in streptozotocin-induced diabetic neuropathy in male rats (mmol LG^{1})

	Animal groups					
Phospholipids	Normal control	Diabetic acid	Normal+"-lipoic acid	Diabetic+"-lipoic acid	Diabetic+insulin	Diabetic+"-lipoic acid+insulin
PE four week	0.038±0.003^{a}	0.044±0.002^{a}	0.036±0.003^{a}	0.041±0.001^{a}	0.045±0.005^{a}	0.042±0.005^{a}
PS four week	0.029±0.002^{c}	0.033±0.002^{a,b,c}	0.037±0.006^{a,b,c}	0.041±0.004^{a,b}	0.044±0.001^{a}	0.032±0.003^{b,c}
PC four week	0.024±0.002^{a}	0.028±0.002^{a}	0.024±0.002^{a}	0.027±0.0001^{a}	0.027±0.002^{a}	0.027±0.003^{a}
PG four week	1.818±0.086^{a}	1.546±0.122^{a}	1.559±0.112^{a}	1.707±0.083^{a}	1.793±0.096^{a}	1.737±0.046^{a}
SP four week	0.047±0.006^{a,b}	0.054±0.005^{a}	0.038±0.004^{b,c}	0.028±0.001^{c,d}	0.027±0.0001^{d}	0.028±0.0001^{c,d}
PE six week	0.044±0.002^{a,b}	0.046±0.004^{ab}	0.039±0.004^{b,c}	0.048±0.001^{a}	0.034±0.002^{c}	0.040±0.003 ^{a,b,c}
PS six week	0.031±0.003^{b}	0.038±0.004^{a,b}	0.027±0.004^{b}	0.047±0.006 ^{a}	0.030±0.002^{b}	0.030±0.002^{b}
PC six week	0.030±0.001^{a,b}	0.027±0.002^{b,c}	0.023±0.0001^{c,d}	0.032±0.001^{a}	0.022±0.001^{d}	0.022±0.002^{d}
PG six week	1.129±0.04^{b,c}	1.548±0.073^{a}	1.275±0.081^{b}	1.263±0.094^{b}	1.133±0.039^{b,c}	1.044±0.010^{c}
SP six week	0.048±0.002^{a,b}	0.050±0.002^{a,b}	0.045±0.002^{b}	0.058±0.004^{a}	0.053±0.05^{a,b}	0.043±0.004^{b}

PE: Phosphatidylethanolamine, PS: Phosphatidylserine, PC: Phosphatidylcholine, PG: Phosphatidylglycerol, SP: Sphingomyelin, Data are represented as ø± SE ø: Mean values, SE: Standard error, Mean values with different superscript letters in the same column are significantly different at p#0.05

phosphatidylglycerol with significant decreased in phosphatidylcholine with mild decrease in phosphatidylglycerol contents were observed in sciatic nerve of streptozotocin-induced diabetic neuropathy in rats.

The phospholipid composition changes in the peripheral nerve system related to diabetes mellitus and the insulin effect on those changes were investigated by Driscoll *et al*. (1996). Who showed that, more than 92% of the rat sciatic nerve is composed of six major phospholipids: Ethanolamine plasmalogen, phosphatidylethanolamine, phosphatidylserine, sphingomyelin and phosphatidylcholine. This is consistent with previous studies on human sciatic nerve (Driscoll *et al*., 1994) and rat sciatic nerve (Klein and Mandel, 1976). The remaining phospholipid composition consists of five minor phospholipids: Phosphatidic acid, unknown, lysophosphatidylcholine, phosphatidylinositol and alkylacylglycerophosphocholine. Ethanolamine plasmalogen was the most prominent phospholipids detected in all sciatic nerves, average ranging from 32.94% in insulin treated rats to a low of 29.08% in the untreated diabetic group. It has been shown that ethanolamine plasmalogen is important for calcium transport because of the calcium ATPase function in nerve (Gross, 1985). When comparing untreated diabetic rats to insulin treated and control rats, there was a significant decrease in the ethanolamine plasmalogen in the untreated diabetic group Driscoll *et al*. (1996). Phosphatidylcholine is considered the most abundant glycerophospholipid of mammalian tissue (Longmuir, 1987). It assumes a lamellar configuration which stabilizes the membrane that prevents translocation of molecules across the cell membrane (Cullis *et al*., 1987). Therefore, cell membranes with a high portion of phosphatidylcholine will be less permeable. A decreased permeability in the nerve tissue may result in a decreased release and uptake of neurotransmitters at the level of synaptosomal membrane (Greene *et al*., 1988) and in a decrease of the impulse transmission speed (Lithosch and Fain, 1987; Driscoll *et al*., 1994) suggests that, phosphatidylserine replaces the phosphatidylcholine's cell membrane role in the nerve system, as a stabilizer of the bilayer structure due to its lamellar configuration. Phosphatidylserine is a potent activator of protein kinase C (Kutchai, 1993) which is important in the regulation of the phosphocholine cytidyltransferase but it does not intervene in the regulation of the ethanolamine cytidyltransferase (Scherphof, 1993). The results observed here suggest that insulin inhibition of protein kinase C, inhibits the phosphocholine cytidyltransferase, diminishing the phosphatidylcholine synthesis in nerve tissue. The presence of phosphatidic acid and lysophosphatidylcholine in the sciatic nerve indicates phosphatidylcholine hydrolysis, mediated by the enzymatic reaction of phospholipase D and A respectively. There are several indications that certain factors induce the phosphatidylcholine hydrolysis in intact cells: Vasopressin in hepatocytes (Bocckino *et al*., 1985), bradykinin and purinergic agonists in endothelial cells (Martin and Michaelis, 1989) and insulin activity on myocytes (Farese *et al*., 1988). Further hydrolysis occurs as a consequence of mobilizing Ca^{2+} (Navarro *et al*., 1984), or alkylacyl derivative synthesis from phosphatidylcholine in hepatocytes (Augert *et al*., 1989).

Injection of "-lipoic acid to the diabetic rats group caused a non significant increase in the contents of phosphatidylethanolamine, phosphatidylserine and sphingomyelin with significant increase in phosphatidylcholine, followed by significant decreased in the contents of phosphatidylglycerol after four weeks of the treatment. On the other hand, six weeks treatment with "-lipoic acid in diabetic rats caused non significant decrease in the percentage of phosphatidylethanolamine and phosphatidylcholine with significant decrease in the content of sphingomyelin in addition to a non significant increase in the percentage of phosphatidylserine and phosphatidylglycerol when compared with the diabetic non-treated group.

Sphingomyelin is a lamellar phospholipid under physiological conditions and occupies the outer layer of the cell membrane. It serves to "tighten up" the membranes and creates a solid barrier against the invasion of microorganisms or harmful toxic compounds (Svennerholm *et al*., 1992). In nerve tissue it also serves as an insulator ensuring efficient nerve transmission. The relative proportion of this phospholipid and phosphatidylcholine are considered relatively constant for most tissues at approximately one-half of the total phospholipids (Longmuir, 1987; Driscoll *et al*., 1996) concluded that, ethanolamine plasmalogen is the most prominent phospholipids in the nerve membrane with phosphatidylserine which suggests that for the nerve system the typical phosphatidylethanolamine-phosphatidylcholine tandem is replaced by ethanolamin plasmalogen-phosphatidylserine tandem. The general higher levels of choline-containing phospholipids and the lower level of ethanolamine-containing phospholipid, calculated by the ratios, detected in the diabetic group when compared with the non-diabetic and insulin treated groups corroborate the findings in human diabetic and non-diabetic peripheral nerve tissue. Who added that, the rat model for the insulin activity on the sciatic nerve supports the theory that phosphatidylcholine metabolism is regulated and maintained in low concentration as a consequence of the insulin inhibitory effect on protein kinase. There are more ethanolamine phosphoglycerides, (28-39%) than those with choline and plasmalogens (phosphatidylcholine and phosphatidylethanolamine) are reported to be significantly abundant in the peripheral nervous system. Sphingomyelin is more enriched in peripheral nerve myelin, where it represents 10-35% of the total lipids, than in brain myelin, where it accounts for only 3-8% of the lipids (Norton and Cammer, 1984). Impairment of Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase activities in microsomes and altered in oxidative energy metabolism in mitochondria in diabetic state have been demonstrated (Dogru *et al*., 2005; Franzon *et al*., 2005). It is possible that the altered membrane lipid/phospholipid milieu which we report here could be a factor contributing to the observed functional changes (Moreira *et al*., 2004).

Four weeks treatment with insulin alone to diabetic rats significantly decreased the percentage of phosphatidylethanolamine, phosphatidylcholine and phosphatidyglycerol and non significantly decrease the percentage of phosphatidylserine, with non significant increase in the percentage of sphingomyelins. However, six weeks treatment with insulin in diabetic rats resulted in non significant increased the percentage of phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol. While a non significant decrease in the percentage of phosphatidylcholine and significant decreased in the percentage of sphingomyelin were observed when compared with diabetic non treated group.

Similarly, Patel and Katyare (2006) reported that, in early diabetic stage there was increase in the sphingomyelin (SPM) while phosphatidylinositol (PI) and phosphatidylserine (PS) components decreased. Insulin treatment restored SPM and decreased the lysophospholipids (Lyso), PI, PS and phosphatidic acid (PA); phosphatidylethanolamine (PE) increased. In 1 month diabetic group phosphatidylcholine (PC) decreased while PI, PS and PE increased. Insulin treatment lowered the Lyso. SPM, PI, PS and PA while PC and PE increased. Who added that, in mitochondria, at early stage of diabetes both CHL and TPL contents decreased; insulin treatment restored the former component. Late diabetic stage had no effect on CHL and TPL contents; insulin treatment brought about reduction in both. Diabetic state had marginal effect on phospholipid composition at both the stages. Insulin treatment had a generalized effect of lowering of PI and PS components and increasing diphosphatidylglycerol (DPG). Moreover, In 1 month diabetic animals the PI, PS and PE components increased and the PC component decreased. Insulin treatment

caused significant decrease in Lyso. SPM, PL PS and PA whereas PC and PE registered significant increase who also added that, insulin treatment resulted in increase in the two basic phospholipids, viz., PC and PE while lowering SPM. Additionally, insulin treatment had adverse effect on the content of acidic phospholipids, viz., PI and PS. This effect was especially pronounced in 1 month group where diabetic state had elevated the PI and PS components.

Moreover, Driscoll *et al*. (1996) showed that, in diabetic rats phosphatidylcholine was significantly elevated and ethanolamine plasmalogen and choline plasmalogen were significantly lower when compared with both control and insulin treated rats. The choline ratio (choline-containing phospholipids over noncholine phospholipids) was significantly elevated in the diabetic group, when compared with both control and insulin-treated groups. The ethanolamine ratio (ethanolamine-containing phospholipids over nonethanolamine phospholipids) and the ratio of the ethanolamine ratio over the choline ratio, was significantly elevated in the control and the insulin-treated groups when compared with the diabetic group. The presence of phosphatidic acid and the significance in phosphatidylcholine and ethanolamine plasmalogen, suggested that insulin had a role in the phosphatidylcholine metabolism in the rat nerve. Several phospholipases have been reported to catalyze the degradation of phosphatidylcholine to liberate choline in the synthesis of acetylcholine. Phospholipase A_1, A_2 and lysophospholipases generate glycero-3-phosphorylcholine which can subsequently be hydrolyzed to yield free choline. This choline, under the action of the choline-acetyltransferase, reacts with acetyl-CoA to synthesize acetylcholine (Blusztajn and Wurtman, 1981; Crews (1987) suggested that, transmethylation plays a role in providing a source of choline for acetylcholine. Phospholipid methylation is the only known metabolic route by which de novo choline is formed. In basal fore brain cell cultures, insulin has been shown to increase the activity of cholineacetyltransferase in a dose dependent manner (Knusel and Hefti, 1991).

Four weeks treatment with "-lipoic acid combined with insulin to diabetic group non significantly decrease the percentage of phosphatidylethanolamine, phosphatidylserine and sphingomyelin and significantly decrease the percentage of phosphatidylcholine and phosphatidylglycerol. However, six weeks treatment with "-lipoic acid combined with insulin caused non significant decrease the percentage of phosphatidylethanolamine, phosphatidylserine and phosphatidycholine with significant decrease in the percentage of sphingomyelin and non significant increase in the percentage of phosphatidylglycerol when compared with diabetic non-treated rats group. Similarly, Patel and Katyare (2006) reported that, phospholipid composition of microsomal membranes in early diabetic rats showed increase in the sphingomyelin (SPM) component with simultaneous decrease in the phosphatidylinositol (PI) and phosphatidylserine (PS) components. Following insulin treatment, SPM became comparable to control whereas PI, PS components decreased further. Insulin treatment also resulted in lowering the proportion of lysophospholipids (Lyso) and Phosphatidic Acid (PA) components. Under these conditions, the phosphatidylethanolamine (PE) component increased significantly with only marginal changes in phosphatidylcholine (PC). However, in 1 month diabetic animals the PI, PS and PE components increased and the PC component decreased. Insulin treatment caused significant decrease in Lyso, SPM, PI, PS and PA whereas PC and PE registered significant increase. On the other hand, in the mitochondria from 1 week diabetic animals the phospholipids composition was practically unchanged except for a significant reduction in Lyso and a tendency towards decrease in diphosphatidylglycerol (DPG) component. Insulin treatment lowered Lyso and PS while DPG increased significantly with marginal increase in SPM. Moreover, in 1 month diabetic animals also the phospholipid composition was practically unchanged except for a small

reduction in PE. Insulin treatment caused significant reduction in PI and PS components and almost a two-fold increase in DPG (Patel and Katyare, 2006). Who added that, the early as well as late diabetic state created imbalance in the relative proportion of TPL and CHL in cerebral mitochondria as well as microsomes. Insulin treatment was effective in restoring the relative proportion of two lipid classes only in mitochondria.

Diabetic state resulted in significant alterations in phospholipids components of the microsomes at early as well as late stages. Insulin treatment resulted in increase in the two basic phospholipids, viz., PC and PE while lowering SPM. Additionally insulin treatment had adverse effect on the content of acidic phospholipids, viz., PI and PS. This effect was especially pronounced in 1 month group where diabetic state had elevated the PI and PS components. In the mitochondria, diabetic state had only marginal influence on the phospholipid composition and insulin treatment had an acidic phospholipids lowering effect. Also, insulin treatment significantly increased the content of DPG in both the diabetic groups (Patel and Katyare, 2006). Previously it has been shown that the mitochondrial synthesis of DPG in the liver is regulated by thyroid hormones (Hostetler, 1991). Results of our present study would imply that at least in the brain mitochondria, insulin may have regulatory role in DPG biosynthesis. Most of the phospholipids except SPM and DPG are synthesized in the microsomes (Koval and Pagano, 1991).

From the obtained results it is possible to conclude that administrations of alpha lipoic acid and insulin combination in STZ-induced diabetic neuropathy in rats can reduce the risk of metabolic abnormalities responsible for the initial defects in nerve function and contribute to the characteristic structural changes in chronic diabetic neuropathy with a considerable improvement in lipid metabolism and oxidative stress.

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