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Evaluation of selected etiopathogenic and clinical parameters of painful diabetic neuropathy during treatment with alpha-lipoic acid used intravenously at a dose of 600mg daily.

Doctoral thesis

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This project was approved by the local ethics committee in Gdansk

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1. List of Abbreviations

ALA	alpha-lipoic acid
DP	diabetic polyneuropathy
DNPH	2,4-dinitrophenylhydrazine
BC	blood count
K	potassium
Na	sodium
AST	Aspartate aminotransferase
ALT	Alanine aminitransferase
BIL	Bilirubin
Alk	Alkaline phosphatase
GTP	Gamma-glutamyl transpeptidase
TC	total cholesterol
LDL	low density cholesterol
HDL	high density cholesterol
TG	triglicerydes
ESR	erythrocyte sedimentation rate
IASP	International Association for the Study of Pain
CGRP	calcitonin gene-related peptide
VAS	Visual Analog Scale
NPS	Neuropathy Pain Score
PF	Pharmacoeconomy question
PM1	Peroneal nerve proximal point amplitude
PM2	peroneal nerve distal point amplitude
PMCV	peroneal motor nerve conduction velocity
PMDL	peroneal motor distal latency
PFW	peroneal F wave
MM1	median nerve proximal point amplitude
MM2	median nerve distal point amplitude

MMCV	median nerve motor conduction velocity
MMDL	median nerve motor distal latency
MFW	median nerve F wave
MNSA	median nerve sensory amplitude
MNSL	median nerve sensory latency
MNSV	median nerve sensory velocity
PIEV	pain impact on everyday efficiency of work and vital activity
AAT	additional analgesic treatment
OGTT	oral glucose tolerance test
ROI	reactive oxygen intermediates
AGEs	soluble advanced glycation end products
CHAOS	Cambridge Heart Antioxidant Study
GISSI	Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto
MAP kinase	mitogen-activated protein kinase
RAGE	receptor for Advanced Glycation Endproducts
HMF	hydroxyl methyl furfural
AR	Aldose reductase
NADPH	nicotinamide adenine dinucleotide phosphate
ROI	reactive oxygen intermediate
PKC	protein kinase C
TGF-β	transforming growth factor β
UKPDS	United Kingdom Prospective Diabetes Study
DCCT	Diabetes Control and Complications Trial
CGRP	calcitonin gene-related peptide
IASP	International Association for the Study of Pain
TSS	Total Symptom Score
FPG	fasting plasma glucose
HbA1C, A1C	glycated haemoglobin
NGSP	National Glycohemoglobin Standardization Program
PG	postprandial glucose

List of Abbreviations

GDM	gestational diabetes mellitus
GAD	glutaminic acid decarboxylase
ICA	islet cell autoantibodies
IAA	autoantibodies to insulin
QST	quantitative sensory testing
HLA	human leukocyte antigens
HSA	human serum albumin
CSF	cerebrospinal fluid
Hcy	homocysteine

2. Introduction

2.1 Definition and description of diabetes mellitus and painful neuropathy.

The first clear clinical picture of diabetes was given by the Greek physician Aretaeus of Cappadocia[1] (1st century CE).

The breakthrough in the development of diabetology was the discovery of insulin. Frederick Banting and J.J.R. Macleod were awarded the Nobel Prize in Physiology or Medicine in 1923 for the practical extraction of insulin.[2]

Diabetes is a group of metabolic diseases characterized by hyperglycemia. It results from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels[3].

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[1] Leopold, Eugene (1930). "Aretaeus the Cappadocian: His Contribution to Diabetes Mellitus". Annals of Medical History 2: 424–435.

[2] "The Nobel Prize in Physiology or Medicine 1923". The Nobel Foundation.

[3] Classification and Diagnosis of Diabetes, Diabetes Care, Volume 38, Supplement 1, January 2015

Long-term microvascular complications of diabetes include retinopathy , nephropathy, peripheral neuropathy and autonomic neuropathy – all causing different symptoms and dysfunctions (Tab.1).

Microvascular complications	Clinical problems			
retinopathy	vision disturbances	potential loss of vision		
nephropathy	edema	renal failure		
peripheral neuropathy	pain	risk of foot ulcers	amputations	Charcot joints
autonomic neuropathy	gastrointestinal symptoms	genitourinary symptoms	cardiovascular symptoms	sexual dysfunction

Tab 1.Long-term microvascular complications of diabetes

Patients with diabetes develop also so called macrovascular log-term complications – they have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease (Tab.2).

Macrovascular complications	Clinical problems		
atherosclerotic cardiovascular disease	chronic coronary disease	myocardial infarction	sudden cardiac arrest
peripheral arterial disease	intermittent claudication	peripheral ischemia	amputations
cerebrovascular disease	transient ischemic attacks	stroke	

Tab.2. Long-term macrovascular complications of diabetes

In people with diabetes there is a higher incidence of hypertension and abnormalities of lipoprotein metabolism[3].

The huge majority of cases of diabetes fall into two broad etiopathogenetic patterns – type 1 and type 2. Another types are rare. Etiologic classification[3] of diabetes mellitus and other categories of glucose metabolism disorders is shown on table 3.

1. Type 1 diabetes (due to β-cell destruction, usually leading to absolute insulin deficiency)
2. Type 2 diabetes (due to a progressive insulin secretory defect caused by insulin resistance)
3. Gestational diabetes mellitus (GDM) (diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes)
4. Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced diabetes (such as in the treatment of HIV/AIDS or after organ transplantation)

Tab.3. Classification of diabetes mellitus [3]

2.2. Diabetes and redox imbalance.

The common molecular component of both types of diabetes and their complications is redox imbalance at the level of cells and the whole organism[4],[5] - resulting in the development of chronic complications - retinopathy, nephropathy, impaired memory at the level of hippocampus, kidney damage, neuropathy. The cells' redox balance is based on the presence of reducing agents such as reduced glutathione. The glutathione concentration in the liver cell is ~10 mM, ~20 mM in astrocytes, but only ~0.2 mM in neurons[6]. This indicates poorer relative neuron protection. The plasma

[4] Melvin R. Hayden, James R. Sowers. Redox Imbalance in Diabetes. Antioxidants & Redox Signaling. May 2007, 9(7): 865-867.
[5] Maritim AC1, Sanders RA, Watkins JB 3rd. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol. 2003;17(1):24-38.
[6] Kosower NS, Kosower EM. The glutathione status of cells. Int Rev Cytol. 1978;54:109-60.

glutathione content is ~2-4 μM[7].; There are also thioredoxin and glutaredoxin systems apart from glutathione system[8].

2.3. Glutathione, cytoplasm and insulin signalling

In an intracellular environment glutathione levels are high. By enzyme system cytoplasm is able to regenerate the oxidized glutathione and it is a reducing environment due to the presence of glutathione and enzymes performing reduction reactions. In the cell, there are proteins having free thiol groups , some of which are ionized (the cysteine thiolate) - The cell uses the thiol groups located in proteins' tyrosine phosphatases, to regulate cell response to insulin. The binding of insulin to the insulin receptor results in the activation of NADPH oxidase, which generates reactive oxygen species that may influence in a controlled manner thiol residues of phosphotyrosine phosphatases acting as the negative regulator of insulin signaling.

2.4. Phosphotyrosine phosphatase PTP1B and NADPH oxidase

Phosphotyrosine phosphatase PTP1B is an example of intracellular protein that negatively regulate (via the dephosphorylation of the insulin receptor) signaling initiated by the binding of insulin to the receptor and consequently is responsible for the so-called metabolic syndrome development and increase of insulin resistance. Mice lacking the tyrosine phosphatase PTP1B are protected from diet-induced obesity and increased insulin resistance.[9] PTP1B localizes in the endoplasmic reticulum, negatively regulates the signaling of insulin, leptin, and various growth factors that are involved in wound healing, such as VEGF, EGF, PDGF, and TGFb[10]. Activity if PTP1B significantly rises in diabetic neuropathy[11]. Therefore rises the direction of the search for a drug that is a specific inhibitor of PTP1B.

Natural inhibitor of the PTP1B phosphatases comprising the cysteine thiolate in the catalytic center is hydrogen peroxide - H_2O_2. Of the five isoforms of NADPH oxidase an

[7] Michelet F1, Gueguen R, Leroy P, Wellman M, Nicolas A, Siest G. Blood and plasma glutathione measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits. Clin Chem. 1995 Oct;41(10):1509-17.

[8] Holmgren A.Thioredoxin and glutaredoxin systems. J Biol Chem. 1989 Aug 25;264(24):13963-6. Review.

[9] Bence KK1, Delibegovic M, Xue B, Gorgun CZ, Hotamisligil GS, Neel BG, Kahn BB. Neuronal PTP1B regulates body weight, adiposity and leptin action. Nat Med. 2006 Aug;12(8):917-24. Epub 2006 Jul 16.

[10] Zabolotny JM, Kim YB, Welsh LA, Kershaw EE, Neel BG, Kahn BB. Proteintyrosine phosphatase 1B expression is induced by inflammation in vivo. J Biol Chem 2008;283:14230–14241

[11] Dinh T1, Tecilazich F, Kafanas A, Doupis J, Gnardellis C, Leal E, Tellechea A, Pradhan L, Lyons TE, Giurini JM, Veves A. Mechanisms involved in the development and healing of diabetic foot ulceration. Diabetes. 2012 Nov;61(11):2937-47. doi: 10.2337/db12-0227. Epub 2012 Jun 11.

isoform IV (NOX-4), found in the endothelium[12], is the only one not-generating superoxide anion radical directly, and having the ability to two-electron reduction of oxygen molecules, what results in generation of hydrogen peroxide. Perhaps this enzyme in this way participates in the regulation of cellular response to insulin. It is well known that in diabetes NADPH oxidase subunits are overexpressed and activated in vascular endothelium. (Fig. 1.)

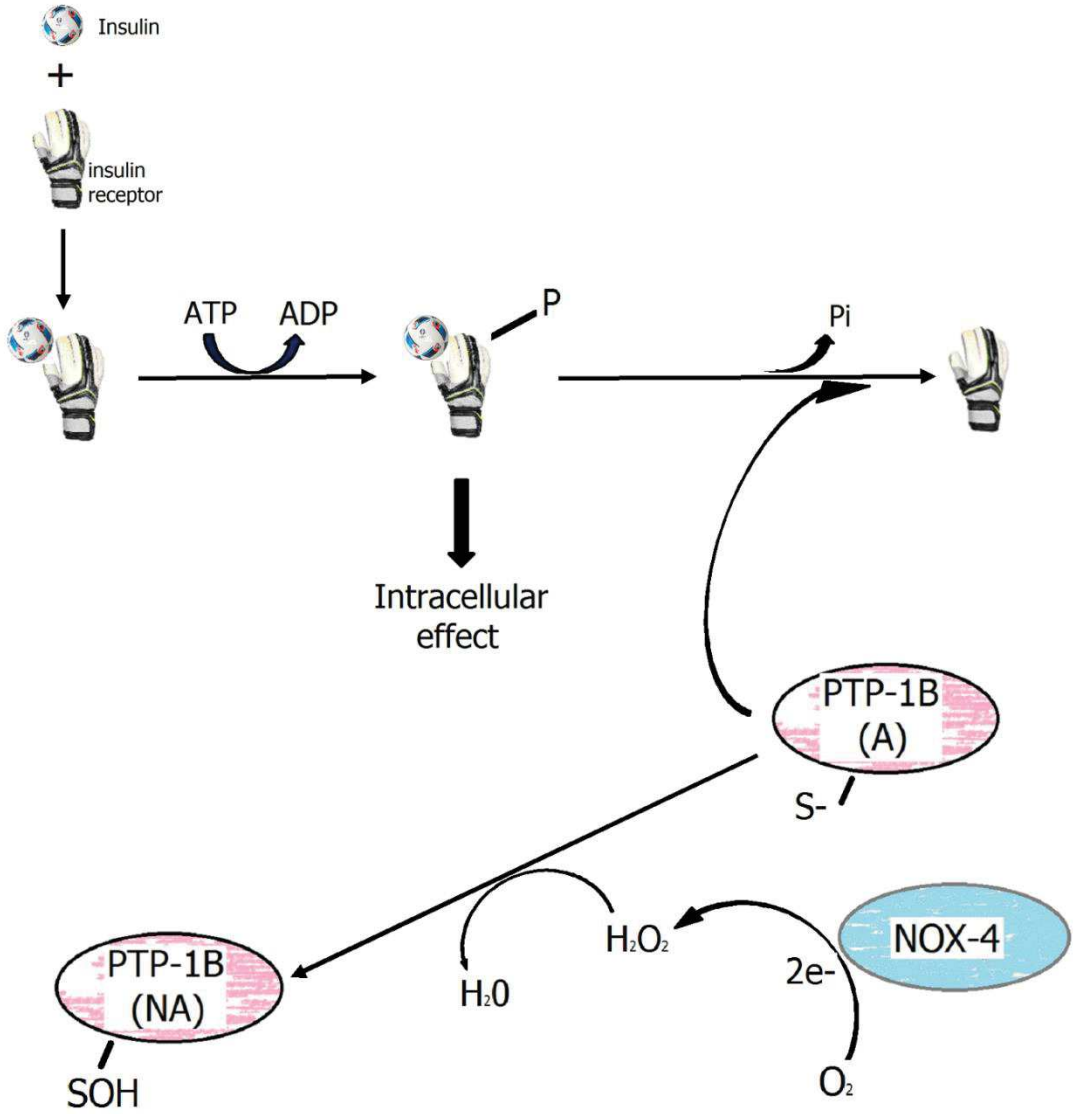

Fig.1. Insulin, PTP-1B and NOX-4 interactions

[12] Kai Chen, Michael T. Kirber, Hui Xiao, Yu Yang, and John F. Keaney, Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. J Cell Biol. 2008 Jun 30; 181(7): 1129–1139.

Alpha-lipoic acid – vitamin, cofactor, drug

Alpha-lipoic acid (ALA) was discovered by Irwin C. Gunsalus, the professor of Biochemistry at the University of Illinois, USA,. ALA[15], which is a cofactor of cellular oxidation processes, once was considered a vitamin – and today we know that it is created in the mitochondria – is an inhibitor of endothelial NADPH oxidase[16],[17] , can improve endothelium function and as is an inhibitor of NADPH. In the blood vessels' wall 5 different isoforms of NADPH oxidase enzymes executing one-electron reduction of molecular oxygen to superoxide anion radical ($O_2^{\cdot -}$) were discovered.

2.5. Albumin – main plasma antioxidant

In contrast to the well-redoxprotected intracellular environment, the concentration of glutathione in plasma is only micromolar, whereas albumin - relatively high (0,66mM). The molecule of albumin is considered to be the main antioxidant in plasma and its structure shows the presence of 17 disulfide bridges and only one 34 cysteine ionized to thiolate, topologically isolated from the strong oxidizing environment of plasma by the location in a sort of "pocket" (Fig.2)[18].

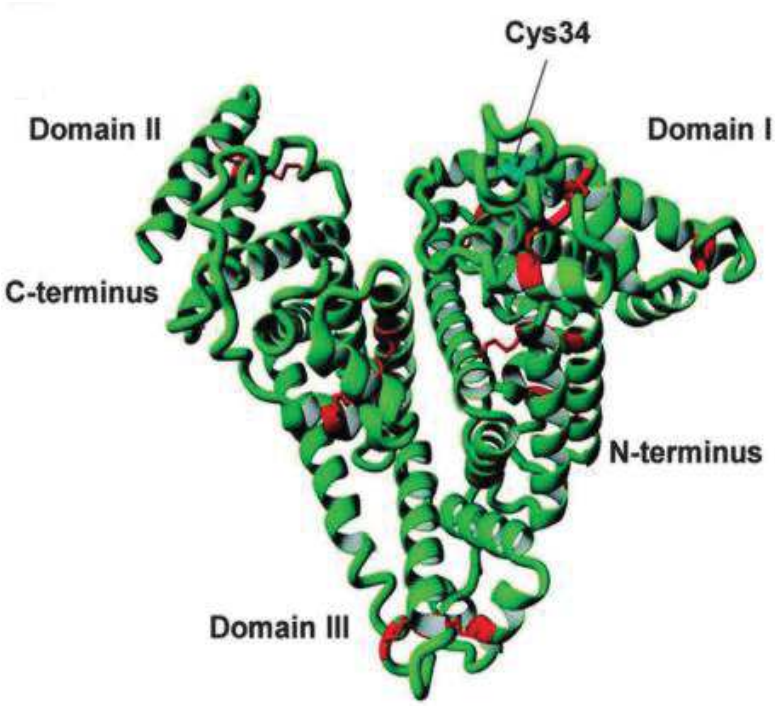

Fig.2 Albumin molecule and the Cys34 pocket.

[13] Coon MJ and Sligar SG (2003). Irwin C. Gunsalus, versatile and creative scientist. Biochem Biophys Res Commun 12: 1–23.
[14] O'kane DJ and Gunsalus IC (1948). Pyruvic Acid Metabolism: A Factor Required for Oxidation by Streptococcus faecalis. J. Bacteriol 56: 499–506.
[15] Prasad PD, Wang H, Huang W, et al. Molecular and functional characterization of the intestinal Na+-dependent multivitamin transporter. Arch Biochem Biophys. 1999;366(1):95-106.
[16] Woo Je Lee, In Kyu Lee, Hyoun Sik Kim, Yun Mi Kim, Eun Hee Koh, Jong Chul Won, Sung Min Han, Min-Seon Kim, Inho Jo, Goo Taeg Oh, In-Sun Park, Jang Hyun Youn, Seong-Wook Park, Ki-Up Lee, Joong-Yeol Park. α-Lipoic Acid Prevents Endothelial Dysfunction in Obese Rats via Activation of AMP-Activated Protein Kinase. Arteriosclerosis, Thrombosis, and Vascular Biology.2005; 25: 2488-2494.
[17] Midaoui AE1, Talbot S1, Lahjouji K1, Dias JP1, Fantus IG2, Couture R1. Effects of Alpha-Lipoic Acid on Oxidative Stress and Kinin Receptor Expression in Obese Zucker Diabetic Fatty Rats. J Diabetes Metab. 2015 Jun 1;6(6):1-7.
[18] Zhibo Liu, Xiaoyuan Chen. Chem. Soc. Rev., 2016,45, 1432-1456. Simple bioconjugate chemistry serves great clinical advances: albumin as a versatile platform for diagnosis and precision therapy. Review Article

2.6. Oxidation of albumin

Cysteine 34 of the human serum albumin is a major pool of SH plasma groups (Fig. 3.).

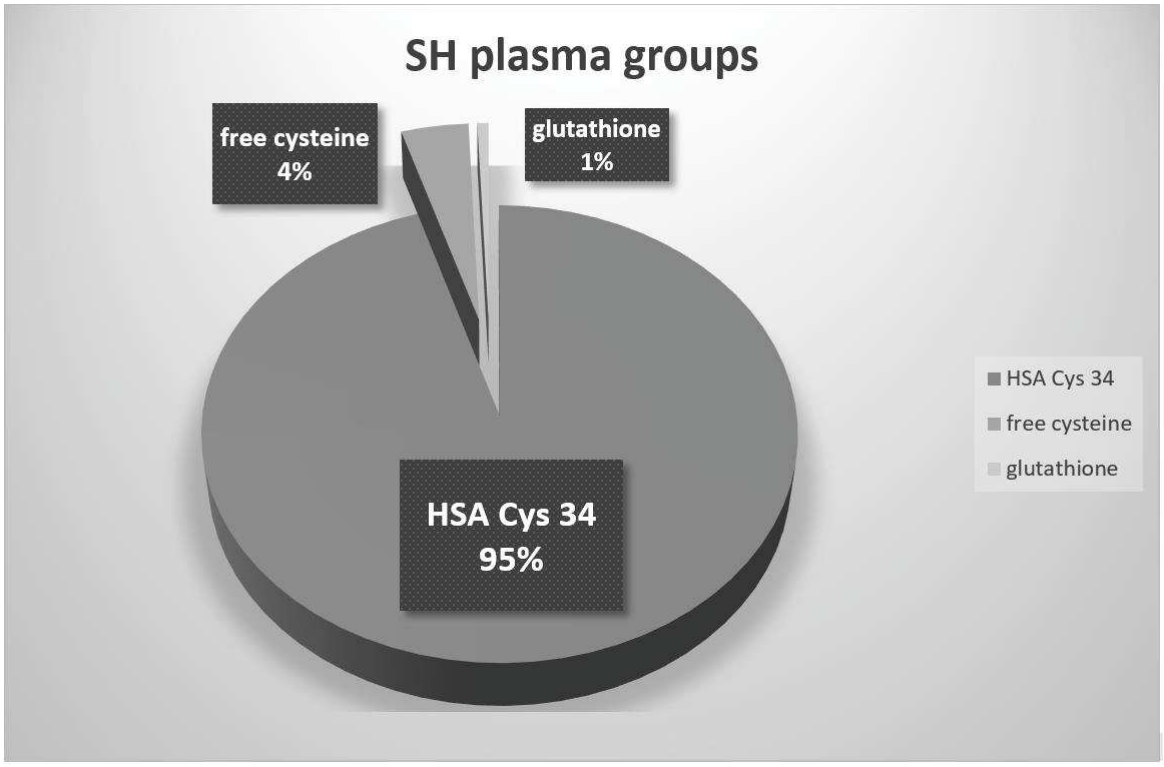

Fig.3. SH plasma groups – Human Serum Albumin cysteine 34 ~700μM, free cysteine ~30μM, glutathione ~5μM[19]

Tests carried out on the redox status of albumin in diabetic patients[20],[21] show a significant degree of cysteine34 oxidation to the sulfenated (SOH), or sulfinic rest(SO_2H).

The basis of this phenomenon is a structural change occurring in the molecule of albumin, depending on albumin molecule binding to more than two fatty acid residues. (Fig 4.). The binding of three fatty acid residues results in a local change of the conformation of the N-terminus of albumin, isomerization cis-trans of proline and

[19] Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism.Annu Rev Nutr. 2004;24:481-509.
[20] A. Guerin-Dubourga, b, A. Catana, E. Bourdona, P. Rondeaua. Structural modifications of human albumin in diabetes. Diabetes & MetabolismVolume 38, Issue 2, April 2012, Pages 171–178.
[21] Oettl K1, Redox state of human serum albumin in terms of cysteine-34 in health and disease.Marsche G.Methods Enzymol. 2010;474:181-95. doi: 10.1016/S0076-6879(10)74011-8. Epub 2010 Jun 20.

cysteine 34, resulting in eversion of the cysteine 34 thiol group to the surface of the molecule into the oxidizing environment containing H_2O_2 and the eventual oxidation.

Oxidized albumin cysteine at position 34 losts its natural antioxidant properties and the ability to bind nitric oxide, a physiological vasodilatator. Hence probably reducing the bioavailability of nitric oxide results in impaired microvascular function of peripheral nerves, which may underlie diabetic polyneuropathy.

The hydrogen peroxide oxidizes not only cysteine34 of albumin, but also in reactions with transition metal - iron, copper - generate very toxic hydroxyl radical damaging side chains of amino acids in the carbonyl groups of the albumin.

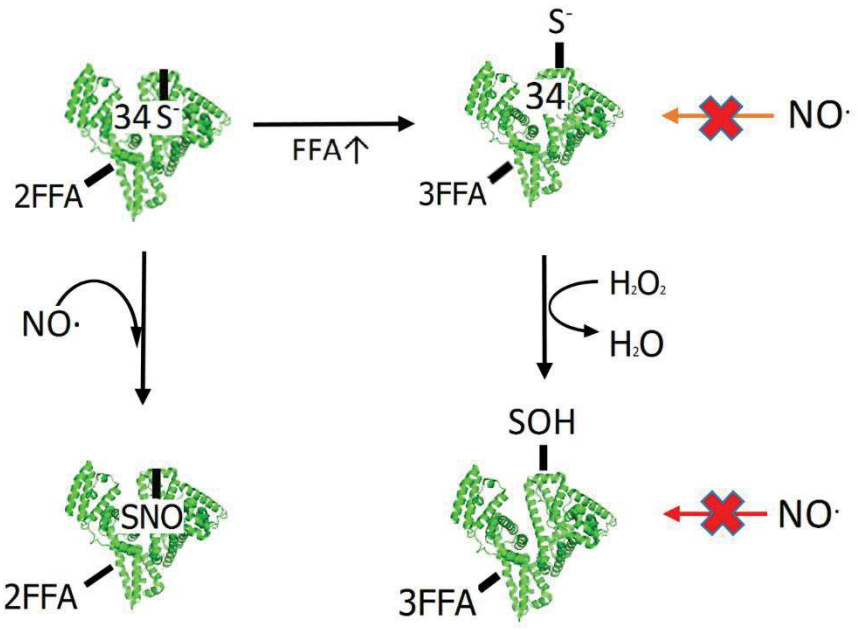

Fig.4. Albumin, FFA and NO·

Attempts to assess the thiol status of albumin are based almost exclusively on the isolation of albumin from plasma[22],[23], platelet rich plasma[24] and assessment of redox

[22] Kawakami A1, Kubota K, Yamada N, Tagami U, Takehana K, Sonaka I, Suzuki E, Hirayama K.Identification and characterization of oxidized human serum albumin. A slight structural change impairs its ligand-binding and antioxidant functions. FEBS J. 2006 Jul;273(14):3346-57.
[23] Ikegaya K1, Nokihara K, Yasuhara T.Characterization of sulfhydryl heterogeneity in human serum albumin and recombinant human serum albumin for clinical use. Biosci Biotechnol Biochem. 2010;74(11):2232-6. Epub 2010 Nov 7.
[24] Giustarini D1, Lorenzini S, Rossi R, Chindamo D, Di Simplicio P, Marcolongo R.Altered thiol pattern in plasma of subjects affected by rheumatoid arthritis.Clin Exp Rheumatol. 2005 Mar-Apr;23(2):205-12.

status of Cys34 after isolation. These methods do not prevent rapid oxidation of albumin, which lowers the results od SH-groups. Some papers give low weird values, that are an artifact and mistaken evidence of non present severe illness. However, research conducted in the Peter J.Sadler[25] laboratory proves that the source of methodical error is associated with the fact that the human serum albumin is redox stable in plasma, but it is immediately oxidized upon isolation (Fig.5.). Moreover, the different centrifuge setup, not always described, results in big discrepancies.

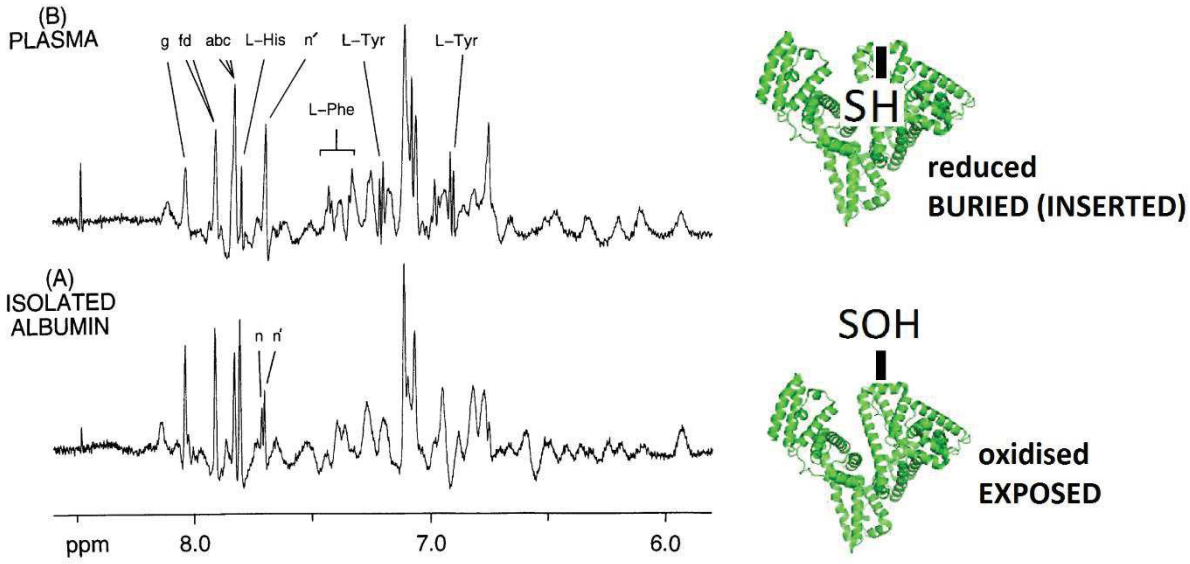

Fig.5. NMR characteristics of reduced(in plasma) and oxidized(after isolation) Cystein45 in Human Serum Albumin. 600 MHz IH NMR spectra of clinically-used (isolated) human albumin (A), (B) fresh human blood plasma. Assignments: n, n' Hsl of His3 of albumin with Cys34 in blocked and thiolate forms, respectively; a to k, other His Hel resonances of albumin; L-Phe, L-Tyr and L-His, free amino acids. Note the high n:n' ratio in (A) compared to (B). Modified with permission of Peter J. Sadler[25]

[25] Christodoulou J1, Sadler PJ, Tucker A. 1H NMR of albumin in human blood plasma: drug binding and redox reactions at Cys34.FEBS Lett. 1995 Nov 27;376(1-2):1-5.

2.7. Albumin and membrane ion channels domain functionality

One of the main molecular biomarkers of polyneuropathy in diabetes is the key body proteins' oxidation. The proteins are serum albumin and potential regulated ion channels present in the membrane of sensory neurons (Tab.4) and endothelial cells' in nerves (vasa nervorum).

Ion channels in sensory neurons:

- involved in signal transduction - response to stimuli :
  - chemical
    - ligand-gated
    - voltage-gated – eq. **Na_v**
  - mechanical
  - thermal
  - chemical/mechanical/thermal – eg. **TRP** – transient receptor potential channels
- involved in the control of excitability
  - background or leak channels
  - Ca^{2+}-dependent channels
  - voltage-gated eg. **T-type Ca_v3.2**

Tab. 4. Ion channels in sensory neurons [26], [27], [28]

A characteristic feature of albumin and transmembrane proteins forming an potential adjustable ion channels conformation is a domain structure. Albumin in the sense of three-dimensional structure can be considered as matching four globular domains , namely: IA, IB = IIA, IIB + IIIA and IIIB, including 10 α-helices, heart-shaped shape.

Domain structure of albumin is responsible for the remarkable ability of the ligand binding, the binding phenomenon of cooperativity and allosteric effects important for the function of the protein.

Potential gated calcium channels of sensory neurons responsible for the generation of pain polyneuropathy also show the presence of four domains located in the plasma

[26] Lee GH1, Kim SS2.Therapeutic Strategies for Neuropathic Pain: Potential Application of Pharmacosynthetics and Optogenetics.Mediators Inflamm. 2016;2016:5808215. doi: 10.1155/2016/5808215. Epub 2016 Jan 13.
[27] Lawrence KrugerMethods in Pain Research. CRC Press, 22.06.2001
[28] Cory Toth, Dwight E. Moulin. Neuropathic Pain: Causes, Management and Understanding. Cambridge University Press, 07.11.2013.

membrane to the cytoplasmic location of the N-terminal and C-terminal. It is worth noting the reduction nature of the intracellular environment and oxidative character of the extracellular environment. The location of four cysteine residues outside the cell is important for channel regulation.(Fig.6.)

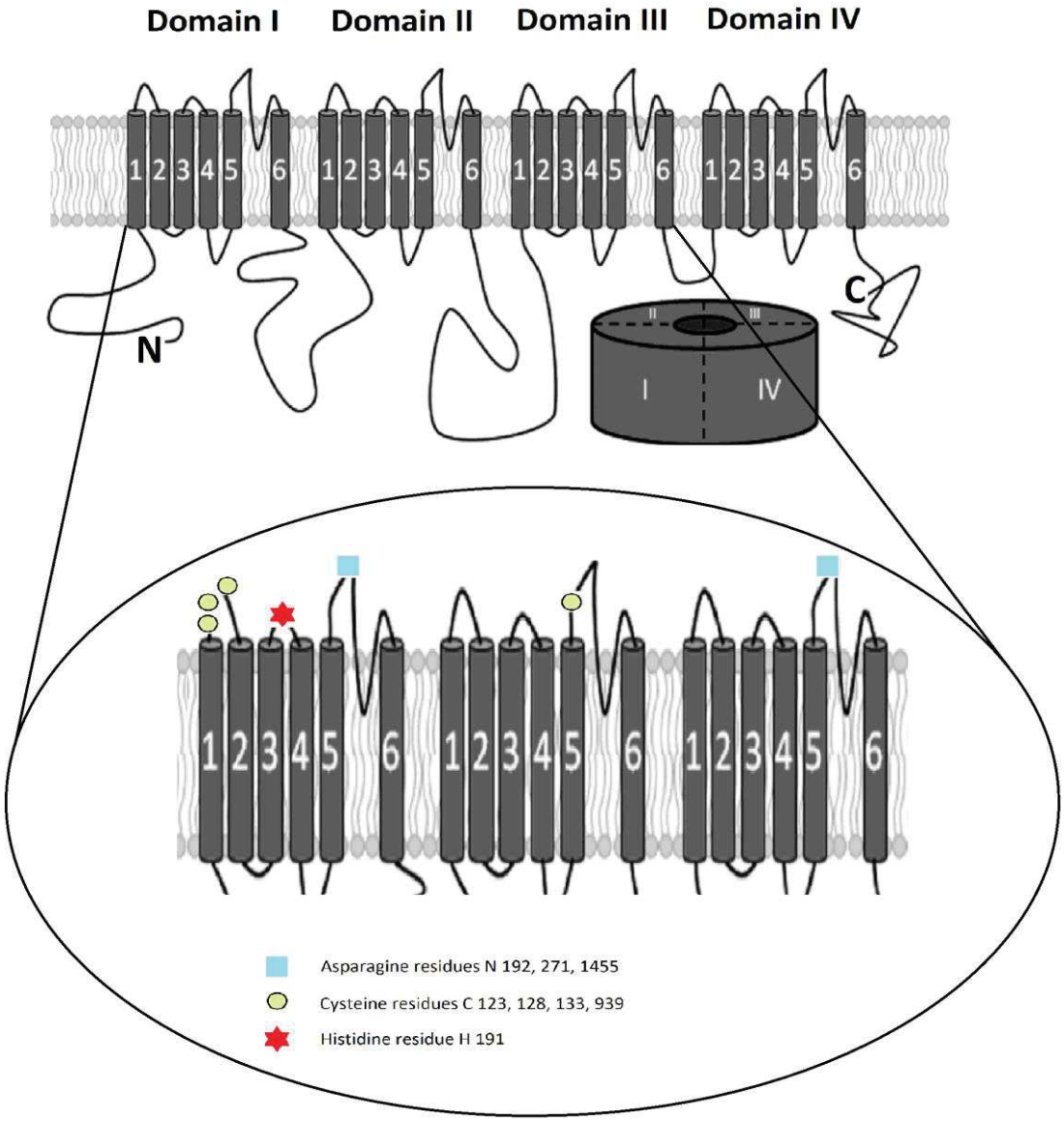

Fig.6. Surface exposed reactive aminoacid residues responsible for physiological activity of $Ca_V3.2$ T-type calcium channels. Asparagine residues N 192, N 1566, responsible for glicosylation process; Cysteine residues C 123, 128, 133, 939, and Histidine residue H 191, responsible for redox processes[29],[30],[31]

[29] Cory Toth, Dwight E. Moulin. Neuropathic Pain: Causes, Management and Understanding. Cambridge University Press 2013
[30] Orestes P1, Osuru HP, McIntire WE, Jacus MO, Salajegheh R, Jagodic MM, Choe W, Lee J, Lee SS, Rose KE, Poiro N, Digruccio MR, Krishnan K, Covey DF, Lee JH, Barrett PQ, Jevtovic-Todorovic V, Todorovic SM. Reversal of neuropathic pain in diabetes by targeting glycosylation of Ca(V)3.2 T-type calcium channels. OrestesDiabetes. 2013 Nov;62(11):3828-38.)
[31] Todorovic SM1, Jevtovic-Todorovic V. Targeting of CaV3.2 T-type calcium channels in peripheral sensory neurons for the treatment of painful diabetic neuropathy. Pflugers Arch. 2014 Apr;466(4):701-6.

2.8. Oxidation of reactive aminoacid residues of receptor domains and albumin

In diabetes, there are redox-mediated changes in the level of oxidation of reactive cysteine and histidine residues which are located paradoxically on the outer surface of receptor domains and albuminThe $Ca_v3.2$ T-type receptor undergoes the cysteine residues bioreduction with the free cysteine, or another poorly understood diabetes bioreductors, resulting in the opening of the calcium channel influx of calcium ions, causing pain sensations by the change of polarization of the sensory neuron cell membrane.

In the treatment of neuropathy lipoic acid works conversely, leading to oxidation of receptor cysteine residues (C123, C128, C133 in domain I and C939 in domain II)[31]

The histidine residues in proteins are particularly susceptible to oxidized transition metal – eg.Fe^{2+}. It has been shown that recombinant human $Ca_v3.2$ channel is susceptible to oxidation of the histidine residue H191 by complex ascorbate-iron and/or copper (IC50 ~ 10 nM)[32],[33]. Oxidation of histidine to 2-OXO-histidine results in closing the channel[34] and analgesic effect. Albumin molecule contains histidine located near the 34 cysteine - both are potentially susceptible to oxidation caused by transient metal[35]. Similar phenomenon may occur in the TRP receptors[36],[37] which are present in neurons, but also in endothelial cells (Fig 7.).

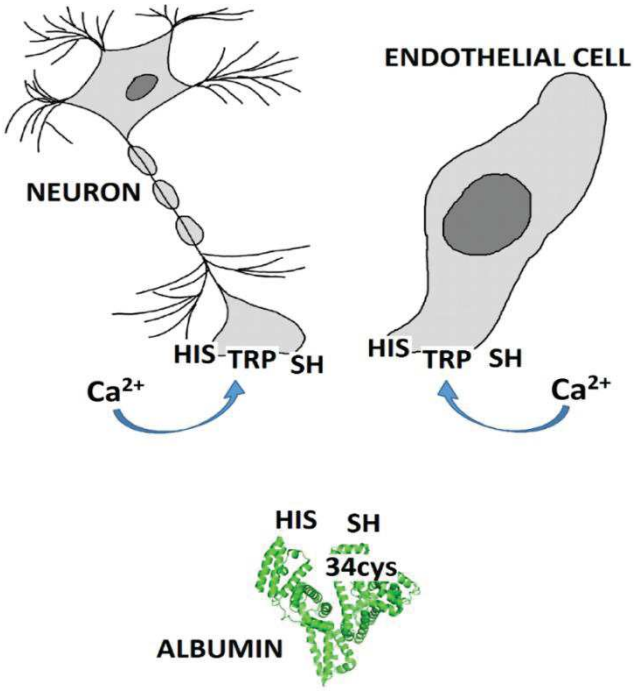

Fig.7. Histidine and SH groups in reaction sites – albumin and TRP receptor of neuron and endothelial cell.

[32] Nelson MT1, Joksovic PM, Su P, Kang HW, Van Deusen A, Baumgart JP, David LS, Snutch TP, Barrett PQ, Lee JH, Zorumski CF, Perez-Reyes E, Todorovic SM.Molecular mechanisms of subtype-specific inhibition of neuronal T-type calcium channels by ascorbate. J Neurosci. 2007 Nov 14;27(46):12577-83.

[33] Kang HW1, Vitko I, Lee SS, Perez-Reyes E, Lee JH.Structural determinants of the high affinity extracellular zinc binding site on Cav3.2 T-type calcium channels. J Biol Chem. 2010 Jan 29;285(5):3271-81.

[34] Slobodan M. Todorovic, Vesna Jevtovic-Todorovic.Redox Regulation of Neuronal Voltage-Gated Calcium Channels. Antioxid Redox Signal. 2014 Aug 20; 21(6): 880–891.

[35] James P. Barnett, Claudia A. Blindauer, Omar Kassaar, Siavash Khazaipoul, Esther M. Martin, Peter J. Sadler, Alan J. Stewart. Allosteric modulation of zinc speciation by fatty acids Biochimica et Biophysica Acta (BBA) - General Subjects, Volume 1830, Issue 12, December 2013, Pages 5456–5464

[36] Dai Y. TRPs and pain. Semin Immunopathol. 2015 Sep 15.

[37] Mickle AD, Shepherd AJ, Mohapatra DP. Sensory TRP channels: the key transducers of nociception and pain. Prog Mol Biol Transl Sci. 2015;131:73-118. doi: 10.1016/bs.pmbts.2015.01.002. Epub 2015 Feb 12.

2.9. Redox chemistry of TRP channels and T-Type calcium channels

Sensation of neuropathic pain can be explained by redox chemistry of TRP channels. These are calcium channels possessing redox-active cysteine, which is sensitive to the presence of reducing and oxidizing agents, eg. nitric oxide activates TRP channels by cysteine S-nitrosylation[38] (involving endotheliocytes) - TRP channels are present both in sensory and motor neurons and endothelial cells.; all contain redox-active cysteine, which regulates the process of opening or closing the channel. It is known that two highly conserved endothelial cells' cysteines 553 and 558 undergo nitrosylation by nitric oxide, what leads to channel opening for calcium and sodium. Similarly $Ca_v3.2$ T-type channels in sensory neurons have four redox-active cysteines which works through the "Reducing Agents"[39] – here the role of alpha-lipoic acid is not to be underestimated(Fig.8.)

Suppression of the channel in vivo brings analgesic effect: in vivo silencing of the cav3.2 T-type calcium channels in sensory neurons alleviates hyperalgesia in rats with streptozotocin-induced diabetic neuropathy[40]

Alpha-lipoic acid influences redox cysteine status[41] of T-type calcium channels thus modulating neuropathic pain.

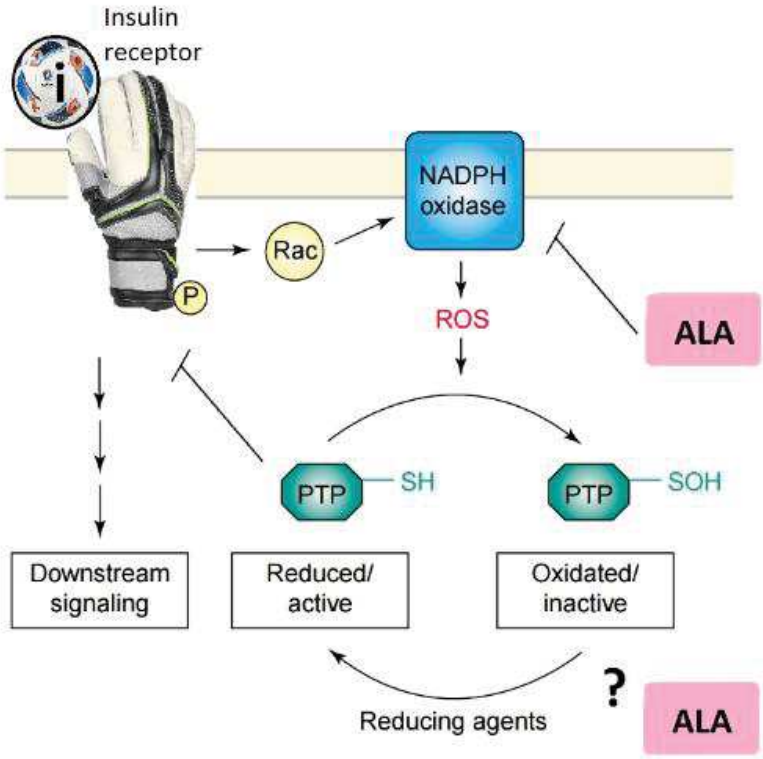

Fig.8. PTP1B, NADPH oxidase, insulin receptor, ALA as reducing agent[39]

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[38] Yoshida T1, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, Tominaga M, Shimizu S, Sato Y, Mori Y. Nitric oxide activates TRP channels by cysteine S-nitrosylation.Nat Chem Biol. 2006 Nov;2(11):596-607.

[39] Chiarugi P1, Cirri P. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction.Trends Biochem Sci. 2003 Sep;28(9):509-14.

[40] Xue-Hong Cao, Hee Sun Byun, Shao-Rui Chen, Hui-Lin Pan. Diabetic Neuropathy Enhances Voltage–Activated Ca2+ Channel Activity and Its Control by M4 Muscarinic Receptors in Primary Sensory Neurons. J Neurochem. 2011 November; 119(3): 594–603.

[41] Woo Yong Lee,¶ Peihan Orestes,¶ Janelle Latham, Ajit K. Naik, Michael T. Nelson, Iuliia Vitko, Edward Perez-Reyes, Vesna Jevtovic-Todorovic, and Slobodan M. Todorovic. Molecular mechanisms of lipoic acid modulation of T-type calcium channels in pain pathway. J Neurosci. 2009 Jul 29; 29(30): 9500–9509.

2.10. Protein glycation

Protein glycation is typical patomechanism of diabetes. Glycated albumin may involve more transition metal ions and hence become subject of unfavorable oxidation. In turn, calcium channel $Ca_v3.2$ in the membrane of sensory neuron exhibits in hyperglycemia phenomenon of glycation of extracellular asparagine residues at positions 192 and 1466 (N192 and N1466). It has been demonstrated that glycosylation of each of these residues results in the opening of the channel and hyperalgesia and allodynia. Deglycosylation induced by neuraminidase reduces these effects (blocking the channel)[31,30](Fig.9)

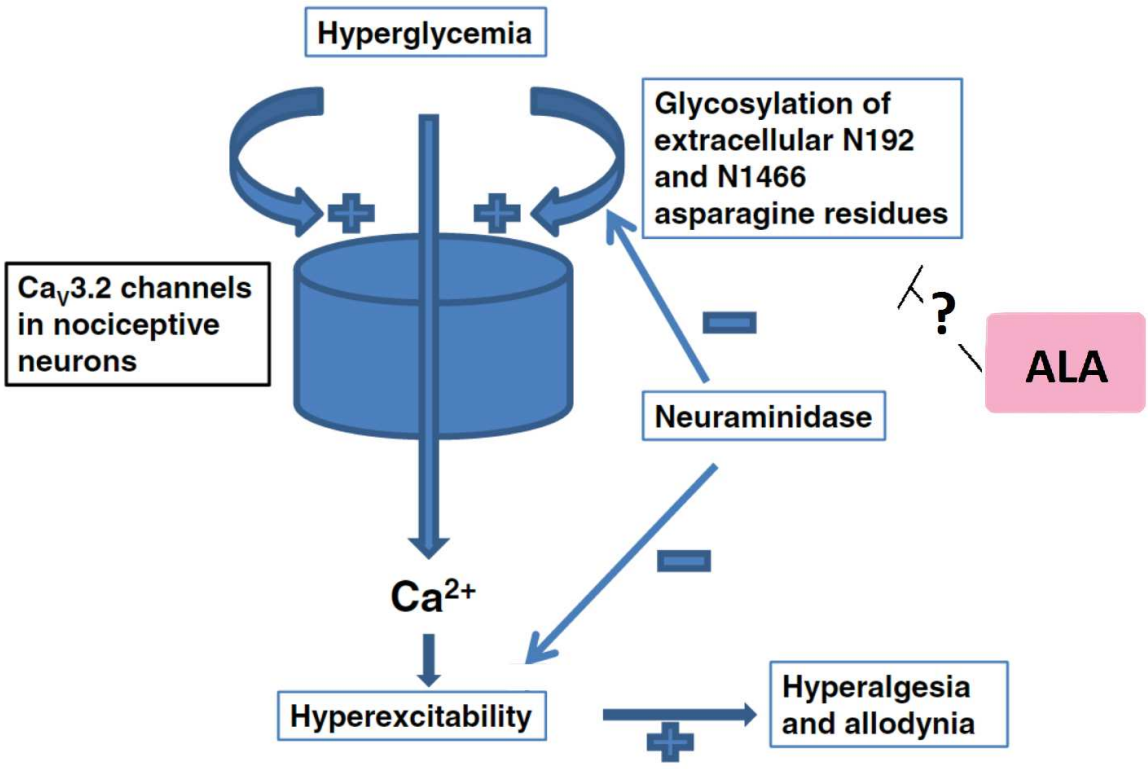

Fig.9. Schematic representation of the effects of glycosylation on $Ca_v3.2$ channels in nociceptive neurons, contributing in painful diabetic neuropathy[31].

2.11. Nitric oxide and cysteine 34 of albumin

It has been shown that a molecule of nitric oxide can be an effective electrophile carrying out the reaction of S-nitrosylation of cysteine residues channel $Ca_v3.2$ T-type[42], resulting in the closure of the passage and analgetic effect in neuropathy. However, the bioavailability of nitric oxide is limited due to the short lifetime and the endothelial nitric oxide synthase expression and function failure under diabetes conditions.

Cysteine 34 of albumin is a nitrogen oxide sensor and after nitrosylation becomes the main source of nitric oxide for both vasodilatation and other receptor-mediated effects.

In conditions of diabetes and metabolic syndrome albumin molecule binds more than 2 fatty acid residues resulting cis-trans isomerization proline-cysteine34, pushing cysteine34 into external environment and oxidation of cysteine[43], limiting the ability to bind to nitric oxide. This reduces the bioavailability of nitric oxide and induces symptoms of neuropathy.

2.12. NADPH oxidase

Another factor limiting the bioavailability of nitric oxide is overexpression and activation of NADPH oxidase in the endothelial cells' membrane. The increased activity of NADPH oxidase provides a product that is superoxide anion which inactivates nitric oxide.

Lipoic acid used in the treatment of symptomatic neuropathy is one of the NADPH oxidase inhibitors[44].

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[42] Jeonghan Lee, Michael T. Nelson, Kirstin E. Rose, and Slobodan M. Todorovic. Redox mechanism of S-nitrosothiol modulation of neuronal CaV3.2 T-type calcium channels. Mol Neurobiol. 2013 Oct; 48(2): 274–280.

[43] María José Torres, Lucía Turell, Horacio Botti, Laura Antmann, Sebastián Carballal, Gerardo Ferrer-Sueta, Rafael Radi and Beatriz Alvarez. Modulation of the reactivity of the thiol of human serum albumin and its sulfenic derivative by fatty acids. Arch Biochem Biophys. 2012 May; 521(1-2): 102–110.

[44] Dong Y1, Wang H1, Chen Z1.Alpha-Lipoic Acid Attenuates Cerebral Ischemia and Reperfusion Injury via Insulin Receptor and PI3K/Akt-Dependent Inhibition of NADPH Oxidase. Int J Endocrinol. 2015;2015:903186. doi: 10.1155/2015/903186. Epub 2015 Jul 29.

3. The Objective of this Paper

The question is whether the presence of alpha-lipoic acid in the vascular bed affects the redox state of cystein-34 of human albumin and the level of oxidation of the amino acid side chains measured using protein carbonyl levels, which may influence albumin's physiological function.

Taking into account that albumin can be a major source of NO and Zn^{2+}, relevant $Ca_v3.2$ channel inhibitors, it is interesting to ask if the possible restoration of its biological function by lipoic acid can affect nerve function.

Then the question arises whether the presence of lipoic acid significantly affects the state of sensory nerve function assessed by testing nerve conduction.

In order to answer these questions it was decided to set up an experiment where the following research questions would be asked:

- whether the administration of intravenous ALA affects redox serum albumin parameters - level of SH groups and plasma protein carbonyls
- what is the effect of ALA on nerve conductivity in the context of the redox status of albumin

To date, no studies have been conducted in patients with diabetic polyneuropathy connecting the main serum antioxidant – albumin - oxidation degree, carbonyl levels and nerve function assessment.

Thus, it seems interesting to examine the redox state of albumin in patients with diabetic polyneuropathy.

The aim of this experiment was to examine the redox status of human albumin in patients with diabetic polyneuropathy in the course of treatment with lipoic acid. Evaluation of the redox albumin was made by measuring the reduced cysteine-34 (using DTNB), and the level of carbonyl groups in patients treated and untreated with lipoic acid.

The degree of control of glucose metabolism in diabetic patients was measured by A1C.

The function of peripheral nerves was assessed on the basis of an electrophysiological study of nerve conduction.

4. Research Design and Methods

4.1 Patients population

The diabetic patients were recruited from APR2005 to NOV2006 from population of Regional Diabetes Center of Gdansk Medical University, Poland. Research took place with the approval of the Local Ethics Comitee. Informed consent was obtained from all subjects eligible to participate in the study. Inclusion criteria were type 1 or type 2 diabetes according to World Health Organisation/American Diabetes Asociation/Polish Diabetes Association criteria, age 20-70 years, clinically evident polyneuropathy, insulin treatment. Exclusion criteria were: 1) non-diabetic neuropathy; 2) smoking or non-smoking<1 year; 3) use of antioxidants (vitamin C, E, probucol, β-karotene, carvedilol, iron chelators, α-lipoic acid) or prooxidants (iron) within the last 3 months; 4) peripheral arterial disease (signs or symptoms); 5) coronary heart disease, myocardial infarction, heart failure, cardiac peacemarker; 6) any medication affecting neurological functions; 7) neurological diseases; 8) blood glucose levels>400mg/dl and/or ketonuria; 9) alcohol abuse; 10) proliferative retinopathy; 11) any systemic insufficiency; 12) any serious disease or instability.

All patients underwent 4 weeks teaching programme including dietary instructions, training in self-monitoring of blood glucose and learning about self-adjustement of insulin dose. Insulin therapy then was optimized with human insulin or insulin analogues in an individual adjustement regarding the fasting and postprandial glycaemias. The treatment phase started after 3 months „stabilisation" period.

Patients were informed that the day before blood sampling they should always have the last light meal no later than at 6 P.M.

Short term glycaemic control was based on self-monitoring of blood glucose, done 6 times daily for a week before treatment, in the first week of treatment, in the third week, and in a week after treatment.

To establish whether the effect of treatment depends on the type of diabetes, disease duration or long term quality of glycaemic control (mean HbA1c) the special analyses were performed. Clinical characteristics of the patients at entry into the study is shown in tab. 4.

4.2 Analytical methods-biochemistry

Self-monitoring capillary blood glucose was determined using the personal Medisense Optium biosensor amperometric glucometers (glucose oxidase GOD test, Abbott Laboratories, Abbott Diabetes Care, Alameda, CA, 94-502 USA). The data was collected with computer programme Medisense Precision Link 2.3.

HbA1c was measured using an ion-exchange HPLC using TOSOH G8 HPLC Analyzer (Tosoh Bioscience, Inc. San Francisco, USA) with a normal range of values 4,3 - 6.1%.

Creatinine serum level was measured by spectrofotometry and urinary albumin excretion by immunoturbidymetry using the ABBOTT ARCHITECT C8000 (Abbott Laboratories, Abbott Park, Illinois, USA)
Additional safety tests included: blood count (BC), potassium (K), sodium (Na), Aspartate aminotransferase (AST), Alanine aminitransferase (ALT), Bilirubin (BIL), Alkaline phosphatase (Alk), Gamma-glutamyl transpeptidase (GTP), total cholesterol (TC), low density cholesterol (LDL), high density cholesterol (HDL), triglicerydes (TG), erythrocyte sedimentation rate (ESR), microalbuminuria.

	Diabetes type 1	Diabetes type 2
Number	13	18
Gender (M/F)	5/8	6/12
Duration of polyneuropathy symptoms –hands (years)	4,1±3,0	2,6±2,6
Duration of polyneuropathy symptoms –legs (years)	5,8±3,5	5,0±2,9
Duration of pain symptoms -hands(years)	2,5±2,4	1,3±2,2
Duration of pain symptoms -legs(years)	6,1±3,4	4,4±3,3
Duration of insulin treatment(years)	17,9±11,6	8,5±5,1
Insulin NPH dose (IU/Day)	20,0±9,2	22,0±12,3
Insulin R dose (IU/Day)	29,0±21,2	18,8±18,7
Insulin R+NPH mixture dose (IU/Day)	40,7±7,0	41,8±14,1
HbA1c (%)	8,6±1,8	7,7±1,1
Age (years)	46,7±7,2	62,8±8,4
Body Mass (kg)	66,7±11,4	83,2±17,0
Height (cm)	169,2±9,6	167,2±10,0
Body Mass Index	23,3±3,6	29,7±5,1
Diabetes mellitus duration (years)	18,0±11,5	16,5±6,5

Tab.4. Clinical characteristics of the patients at entry into the study. Results given as mean ± SEM (range).

Determination of carbonyl groups in oxidized proteins was achieved in plasma as it is described in Levine's[45] method. Blood sample with EDTA was centrifuged 200x g for 20 minutes and 100 µl of supernatant – platelet rich plasma - was aspirated. The sample was incubated for 60 minutes with 100 µl of 20 mM 2,4-dinitrophenylhydrazine (DNPH) solution. Afterwards, the protein was precipitated with 20% trichloroacetic acid. Next, the residue was rinsed 3 times with ethanol and etyl acetate mixture and dissolved in 1 ml 6M guanidine HCl, in temperature 60°C.

Carbonyl content was determined by spectrofotometry, in comparison to blanks, at 370 nm wavelenght, using a molar absorption coefficient (ε) of 22,000 M^{-1} cm^{-1} twice each time, and was expressed as nmol carbonyl/mg protein.

The concentration of carbonyl groups determination started no later than 2 hours after collection. Technical conditions for the collection of blood and signs were identical.

Serum albumin sulphydryl groups were assessed according to Habeeb[46],[47].

4.3 Clinical evaluation

4.3.1 Vital signs

Every patient was clinically examined. Data concerning weight, height, body mass index, gender, were collected. Diagnosis of diabetes mellitus type 1 and type 2 was confirmed according to World Health Organisation/American Diabetes Association/Polish Diabetes Association criteria.

Systolic and diastolic blood pressure were measured using automatic ambulatory blood pressure monitor Omron M-4 Intellisense (OMRON Healthcare Europe BV Kruisweg 577 NL-2132 NA Hoofddorp)

[45] Levine R.L., Garland D., Oliver C.N., Amici A., Climent I., Lenz A.G., Ahn B., Shaltiel S., Stadtman E.R.: Determination of Carbonyl Content In Oxidatively Modified Proteins, Meth. Enzymol. 1990, 186, 464-478.
[46] Habeeb AF. Reaction of protein sulfhydryl groups with Ellman's reagent.Methods Enzymol. 1972;25:457-64
[47] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959 May;82(1):70-7.

4.3.2 Ophtalmic assessment

In order to exclude proliferative retinopathy and describe eventual pathologies ophthalmologic investigation was performed in all patients. This included visual acuity tests, intraocular pressure, and anterior segment estimation by slit lamp (Carl Zeiss SL 115 Classic, Germany 2000). The fundus examination was done following installation of 1% Tropicamid to obtain sufficient mydriasis.

The examination was performed using the non contact slit lamp lens +90D lens (VOLK, Volk Optical, Inc.7893 Enterprise Drive, Mentor, OH 44060, USA).

To obtain retina angiographic visualisation the Topcon fundus camera and Kodak color films were used.

All necessary ophtalmic procedures were performed according to The International Diabetic Retinopathy Division[48].

Optic nerve bunch status - screening for glaucomatous optic nerve damage - was evaluated using confocal scanning laser ophthalmoscopy with Heidelberg Retina Tomograph II (Heidelberg Engineering, Tiergartenstrasse 15, 69-121 Heidelberg, Germany).

4.3.3 Neuropathy symptoms and signs

Neurological examination included:

1) the noting of typical symptoms of polyneuropathy such as pain, paraesthesiae and numbness (at baseline and each visit)

2) examination of tendon reflexes, muscle strength, perception of pin pricks and joint position, vibration perceptron thresholds (VPTs), and monofilament test was measured on a standard matrix (at baseline and each visit)

3) electrophysiologic assessment of peripheral nerve function (at baseline and 6 weeks after treatment)

[48] Early Treatment Diabetic Retinopathy Study Rerearch Group. Classification of diabetic retinopathy from fluorescein angiograms.: ETDRS report number 11. Ophthalmology, 1991, 98 suppl.5, 807-822.

Electrophysiological tests – nerve conduction studies - were performed with an EMG 2000 electromyograph (Schwarzer-Picker, Munich, FRG) using surface electrodes. Motor and sensory nerve conduction velocity (MNCV) was measured in the right median, ulnar and peroneal nerves.

4.3.4 Questionnaires

4.3.4.1 VAS – Visual Analog Scale

VAS - Visual Analog Scale is a useful diagnostic tool[49]. This is a psychometric response scale which is widely used in questionnaires. It is a measurement tool for subjective characteristics or attitudes that cannot be directly measured. When responding to a VAS question, respondents specify their level of agreement to a statement by indicating a position along a continuous line between two end-points. This continuous (or "analogue") aspect of the scale differentiates it from discrete scales such as the Likert scale. For example, the amount of pain that a person feels ranges across a continuum from none to an extreme amount of pain, what can not be tolerated. From the patient's perspective this spectrum appears continuous and their pain does not take discrete jumps, as a categorization of none, mild, moderate and severe would suggest. It was to capture this idea of an underlying continuum that the VAS was devised[49].

The VAS can be compared to other linear scales such as the Likert scale or Borg scale. The sensitivity and reproducibility of the results are broadly very similar, although the VAS may outperform the other scales in some cases[50].

Operationally a VAS is a 100 mm horizontal line, anchored by word descriptors at each end, as illustrated in Fig.10.

No pain/----------/ Intolerable pain

Fig.10. VAS- Visual Analog Scale

[49] Wewers ME, Lowe NK. A critical review of visual analogue scales in the measurement of clinical phenomena. Res Nurs Health. 1990 Aug;13(4):227-36.
[50] S. Grant, T. Aitchison, E. Henderson, J. Christie, S. Zare, J. McMurray, and H. Dargie (1999) A comparison of the reproducibility and the sensitivity to change of visual analogue scales, borg scales, and likert scales in normal subjects during submaximal exercise. doi:10.1378/chest.116.5.1208

The patients are asked to mark on the line the point that represents their perception of their current state. The patient tracks the level of pain on a simple scale.
The VAS score is determined by measuring in millimetres from the left side end of the line to the point that the patient marks.
Observing the changes over time gives practical clear insight of patients pain symptoms[49].

Every patient of studied population was asked to match his own level of pain three times during the study : at the beginning (B), soon after 15 days of therapy with alpha – lipoic acid (F), and a month later (G).

4.3.4.2 Neuropathy Pain Score

Neuropathy Pain Score is a questionnaire consisted with 11 questions, regarding character of pain.

The following questions regards intensity of:

1. strength of pain
2. sharpness sensation
3. burning sensation
4. bruising sensation
5. freezing sensation
6. sensibility sensation
7. itching sensation
8. focal, reccuring pain
9. unpleasantness sensation
10. deep pain sensation
11. superficial pain sensation

Every patient of studied population was asked to answer the questions using the 1-10 scale (minimium and maximum sensation) three times during the study : at the beginning (B), soon after 15 days of therapy with alpha – lipoic acid (F), and a month later (G).

4.3.4.3 Pharmacoeconomy question

Every patient of studied population was asked to approximate, for how many days during last 3 months the neuropathic pain interfered with work. The question was answered three times during the study : at the beginning (B), just after 15 days of therapy with alpha – lipoic acid (F), and a month later (G).

4.4 Study schedule

Study schedule is shown on table 5.

Visit	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		18
			TREATMENT PERIOD																
Day of treatment			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Informed consent	x																		
Teaching programme	x																		
Stabilisation period	x																		
Self-monitoring of blood glucose*		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Clinical assesment	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
Blood tests - carbonyl, SH groups			x (B) day 1																x(G) day 30
Blood tests - biochemistry	x(A)		x (B) day 1														x (F) day 15		x(G) day 30
Blood tests - HbA1c	x(A)																x (F) day 15		
Ophtalmic assesment	x																		
Neurological examination			x (B) day 1																x(G) day 30
Questionnaires			x (B) day 1														x (F) day 15		x (G) day 30

*For the patients' safety, there was self-monitoring of blood glucose, to avoid accidental hypoglycemia/unconsciousness from hypoglycemia

Table 5. Study schedule

4.5 Statistical analysis

Statistical analysis was performed with StatSoft, Inc. (2007). STATISTICA (data analysis software system), version 8.0 and Microsoft Excel calculation sheet. The classical measurements were used: mean, median, range (R) and standard deviation. Normality and homogeneity of investigated features in groups were tested using the Shapiro-Wilk test and variance homogeneity test. In order to compare nonparametric data in both groups U mann-Whitney test was performed, and for normal distribution data t-Student test was used, and Pearson correlations were performed between dependent variables.

In time-changing data assessment with regard for variables distribution character in double comparison Wilcoxon signed rank test was implemented, and in multiple comparison Friedman variance analysis was used. All statistical tests were performed accepting statistical significance at the level of $p<0,05$.

The results are expressed as the mean ± standard error.

4.6 Ethics

This study was approved by The Ethics Committee of The Medical University of Gdańsk NKEBN/924/2004 and the investigation was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2004. All patients gave written informed consent for the study approved by the local Ethics Committee.

5.0. Results

5.1. Population characteristics

Patients population is described in chapter 4.1.

5.2. Biochemical parameters influenced by alpha-lipoic treatment

5.2.1. HbA1c

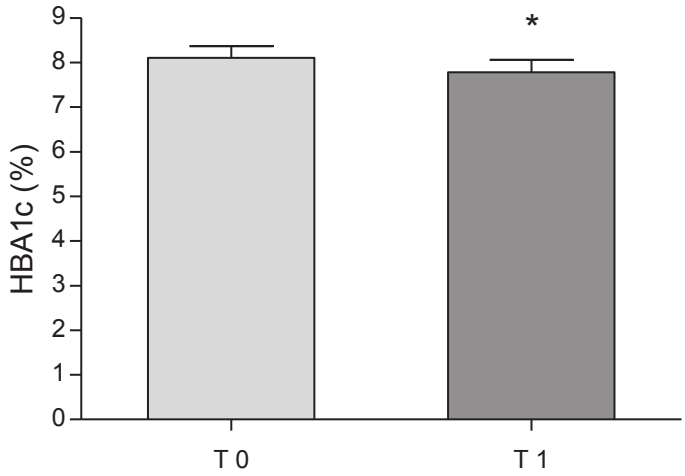

Fig.11. Decrease of HBA1c in the whole studied population with diabetes type 1 and diabetes type 2 (n = 30) and diabetic painful neuropathy (T0-before treatment, T1-after 15 days of treatment with intravenous 600 mg alpha-lipoic acid) – p value derived from Wilcoxon test . (*p<0,05)

HbA1c after treatment with alpha-lipoic acid decreased significantly(p = 0,0012).

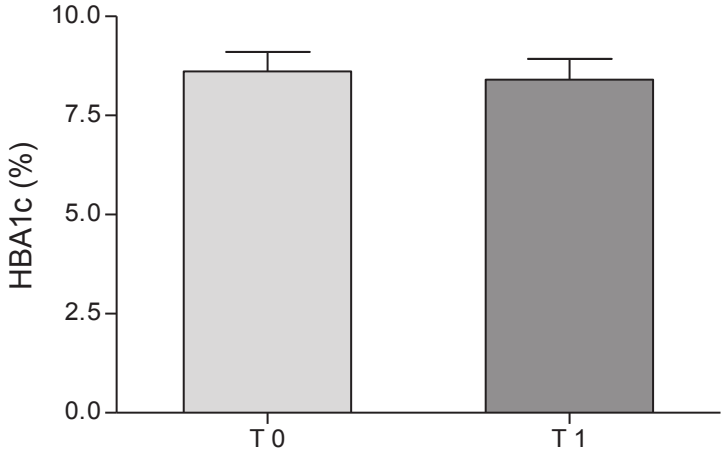

Fig.12. In DM1 group (n = 13), HbA1c decrease was not significant (p = 0,067). (T0-before treatment, T1-after 15 days of treatment with intravenous 600 mg alpha-lipoic acid). Values given in %. Lack of significancy is probably due to insulin dose adjustements, more easy to achieve in good educated diabetes type 1 patients.

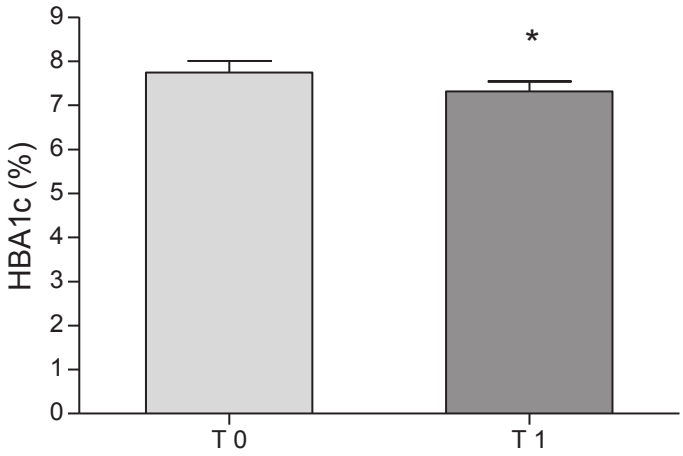

Fig.13. In DM2 group (n = 17), HbA1c decrease was significant (p = 0,0074). (T0-before treatment, T1-after 15 days of treatment with intravenous alpha-lipoic acid). Values given in %. (*p<0,05). The effect is significant probably due to stable insulin dosing.

5.2.2. Blood count

Parameters of blood count (HGB, RBC, HTC, WBC, MCV, MCH, PLT, , neutr%,), measured over time, were not changing.

5.2.3. Blood chemistry

Erythrocyte sedimentation rate, creatinine level, Aspat, Alat were not changing. Falk, GGTP, HDL and triglicerydes did not change. Bilirubin level, total cholesterol and LDL did not change.

5.2.4.Microalbuminuria

	Mean	**Std.**
A -MA	**119,50**	**379,68**
B -MA	**121,49**	**422,32**
F -MA	**177,60**	**667,71**
G -MA	**210,62**	**738,12**

Tab.6. Microalbuminuria was not changing over time (p=0.34 ANOVA Friedman test). (Values given in mg/24h).

5.3. Neurological parameters

All neurological parameters were measured twice: before (T0) and a month after (T1) treatment with alpha-lipoic acid.

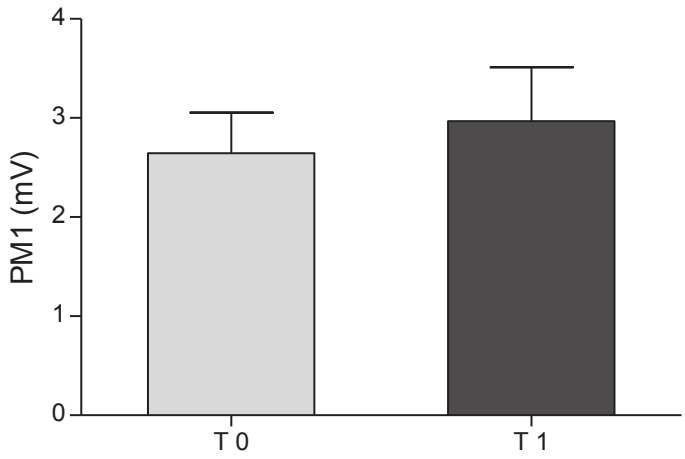

Fig.14. PM1 – peroneal nerve proximal point amplitude after treatment was enhanced – but not significantly (p = 0,68)(values given in mV).

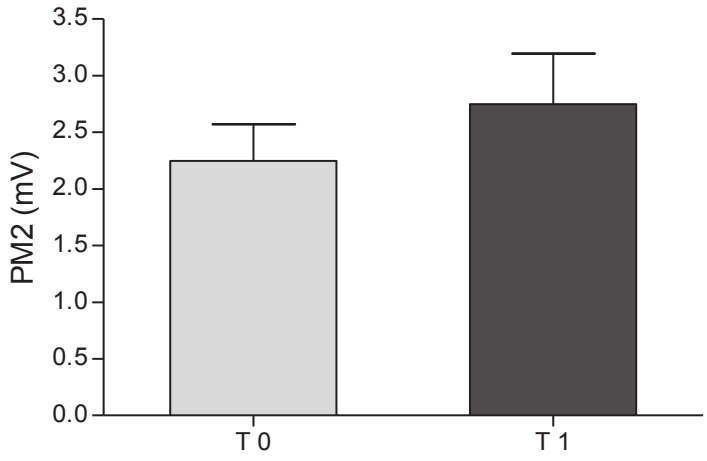

Fig.15. PM2 – peroneal nerve distal point amplitude after treatment was enhanced – but not significantly (p = 0,39)(values given in mV).

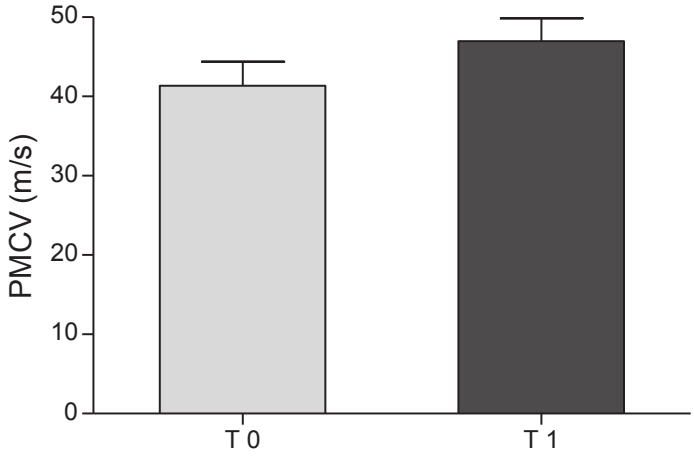

Fig.16. PMCV – peroneal motor nerve conduction velocity enhanced after treatment (p = 0.165) (values given in m/s).

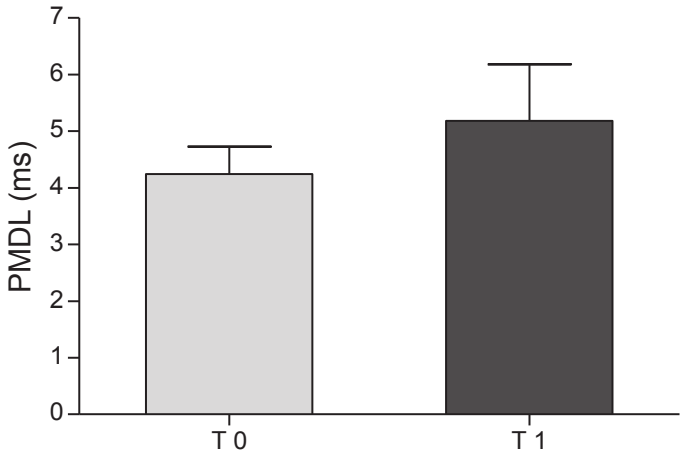

Fig.17. PMDL – peroneal motor distal latency changed not significantly (p = 0.669) (values given in ms).

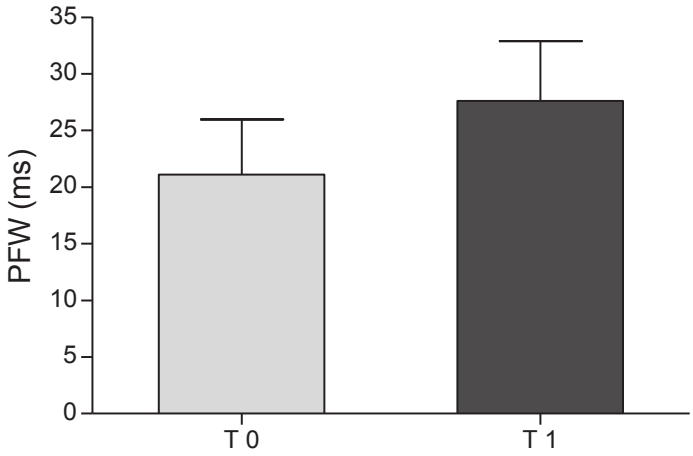

Fig.18. PFW – peroneal F wave – p= 0.615(values given in ms).

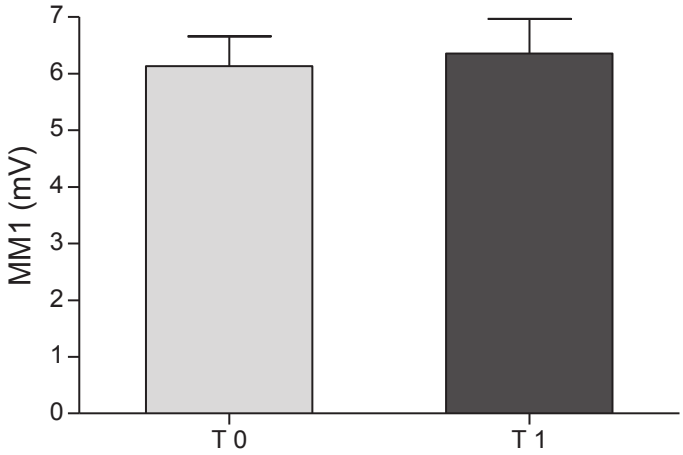

Fig.19. MM1 – median nerve proximal point amplitude (p = 0.95) (values given in mV).

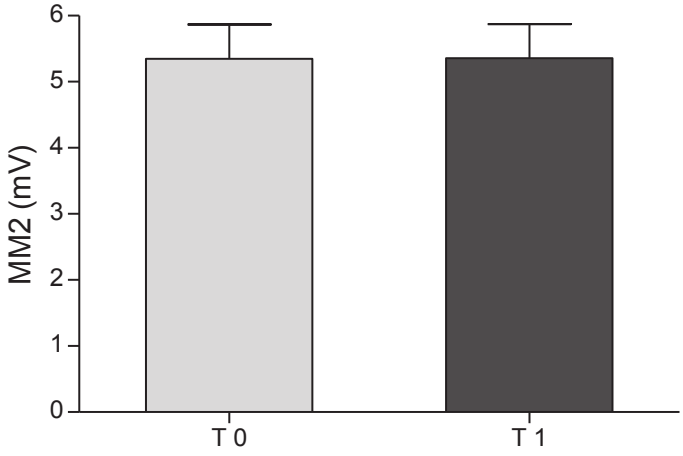

Fig.20. MM2 – median nerve distal point amplitude (p = 0.99) (values given in mV).

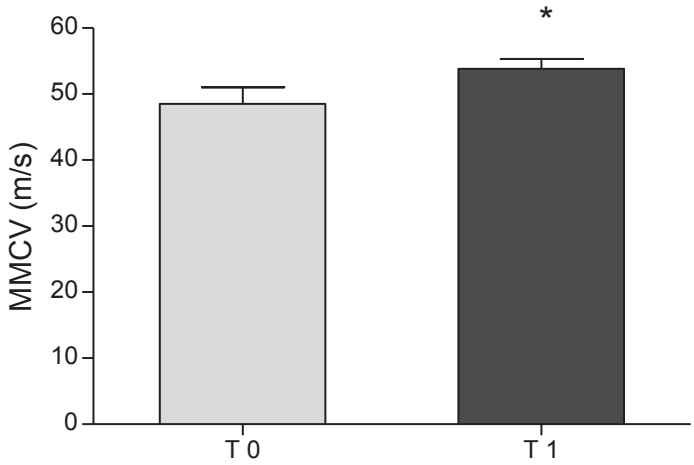

Fig.21. MMCV – median nerve motor conduction velocity improved very clearly (p = 0.15) (values given in m/s).

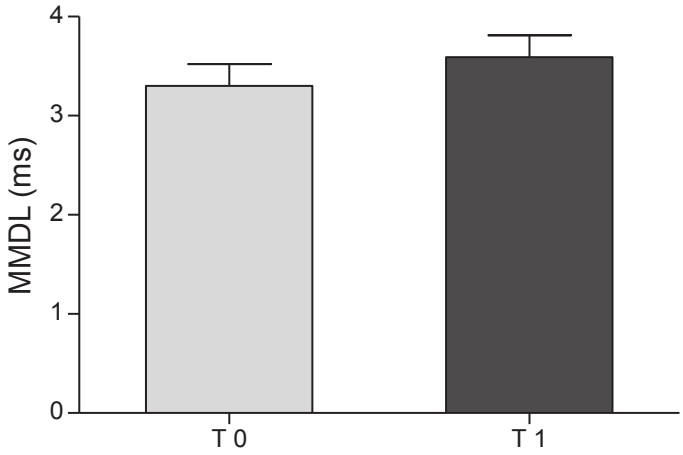

Fig.22. MMDL – median nerve motor distal latency – improvement not significant (p = 0.626) (values given in ms).

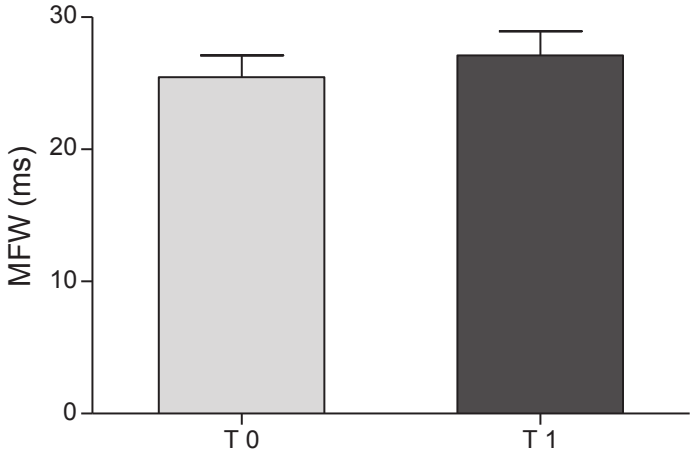

Fig.23.MFW – median nerve F wave – improvement not significant (p = 0.17) (values given in ms).

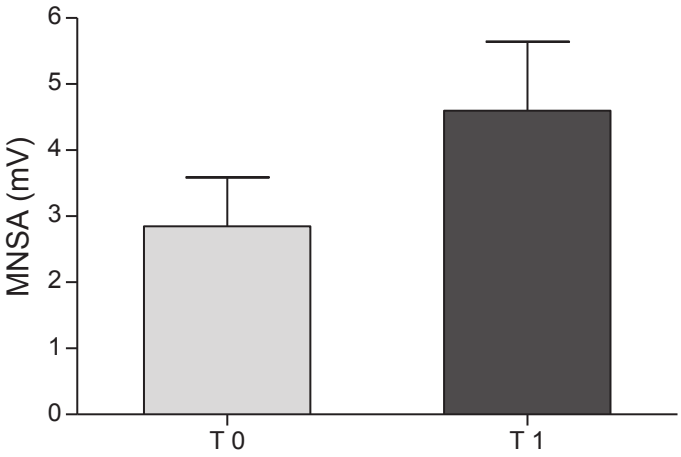

Fig.24. MNSA – median nerve sensory amplitude – improvement not significant (p = 0.33) (values given in mV).

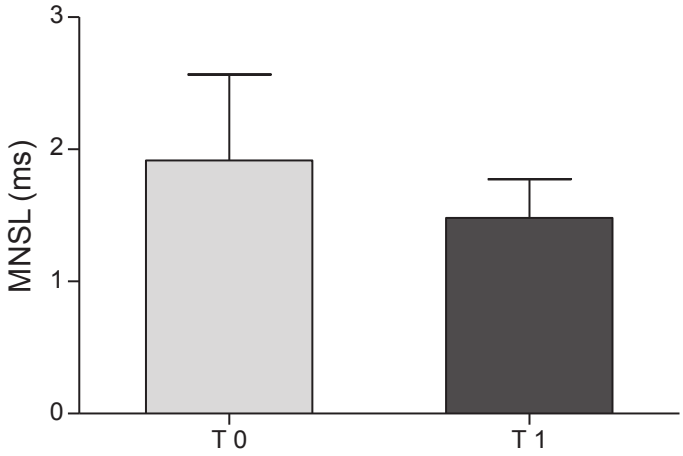

Fig.25. MNSL – median nerve sensory latency – improvement not significant (p = 0.33) (values given in ms).

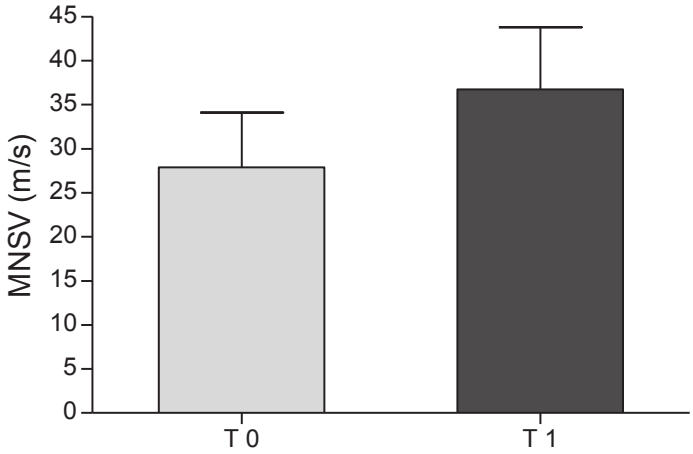

Fig.26. MNSV – median nerve sensory velocity – statistically significant improvement (p = 0.23) (values given in m/s).

5.4. Oxidative stress reactivity during alpha-lipoic acid treatment : plasma proteins' carbonyl, plasma protein and plasma proteins' SH groups

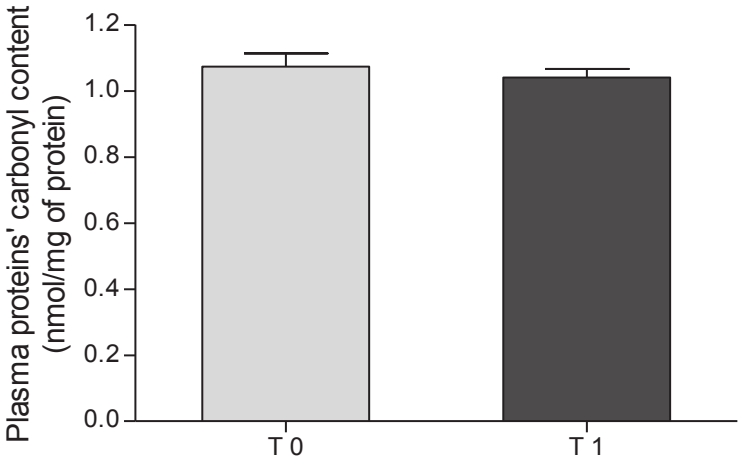

Fig.27. Change in plasma proteins' carbonyl content in the whole population of patients with diabetes type 1 (n=13) and type 2 (n=17) with painful diabetic neuropathy (T0 – before treatment, T1- 14 days after treatment with intravenous 600 mg alpha-lipoic acid) (p = 0,992) (values given in nmol/mg protein).

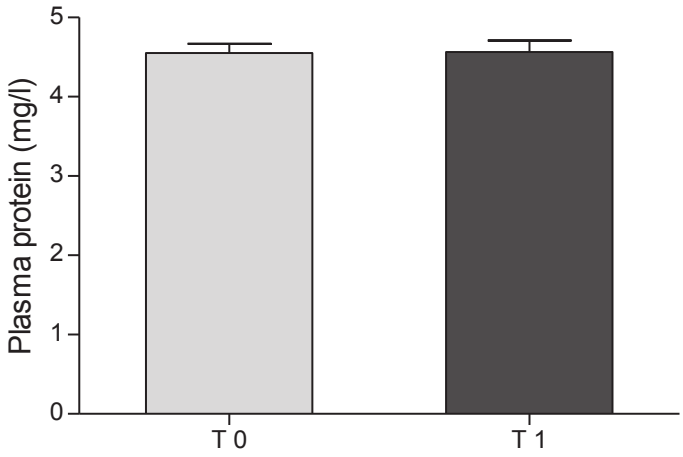

Fig.28. No change in plasma protein after treatment with alpha-lipoic acid (values given in mg/l) (p=0.968).

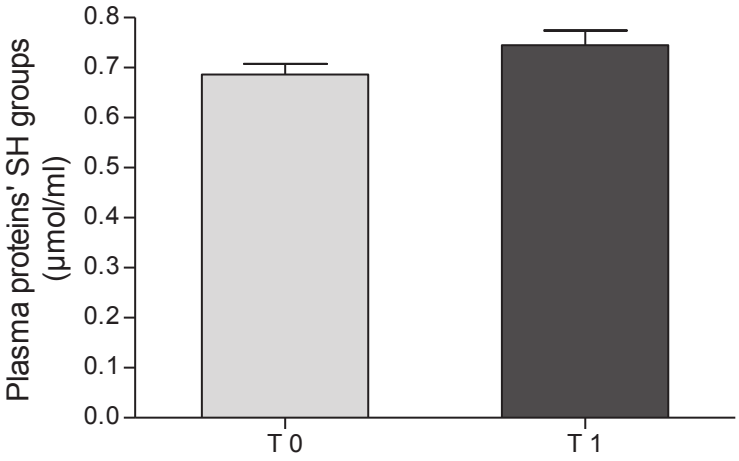

Fig.29. Change in plasma proteins' SH groups after treatment with alpha-lipoic acid (p = 0,11) (values given in µmol/ml of plasma).

5.4.1. Oxidative stress reactivity during alpha-lipoic acid treatment : plasma proteins' carbonyl and plasma proteins' SH groups – relation to diabetes duration

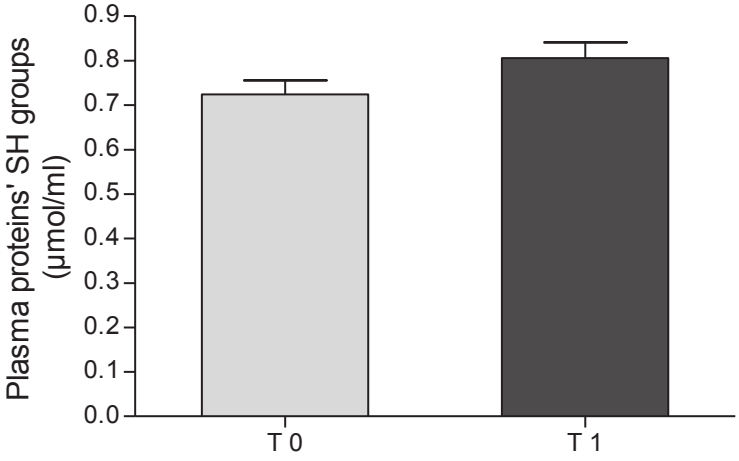

Fig.30. Change in plasma proteins' SH groups in patients diabetes mellitus of shorter duration(<16 years) (p = 0.126) (values given in µmol/ml of plasma).

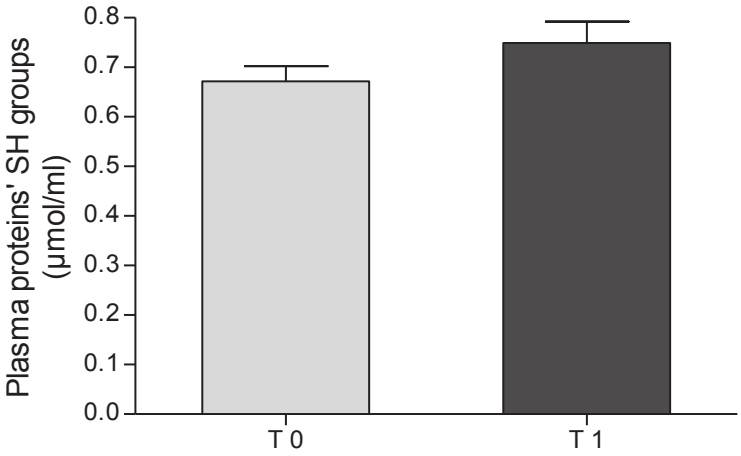

Fig.31. Change in plasma proteins' SH groups in patients with long-lasting (>16 years) diabetes mellitus (p = 0.367) (values given in µmol/ml of plasma).

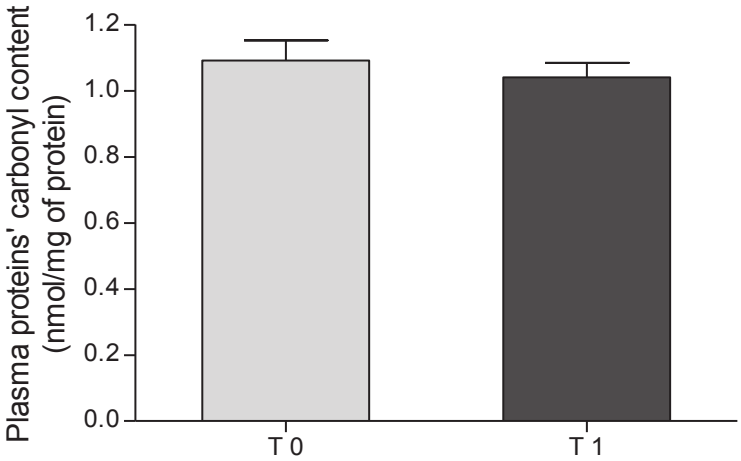

Fig.32. Change in plasma proteins' carbonyl in patients diabetes mellitus of shorter duration(<16 years) (p = 0.811) (values given in nmol/mg protein).

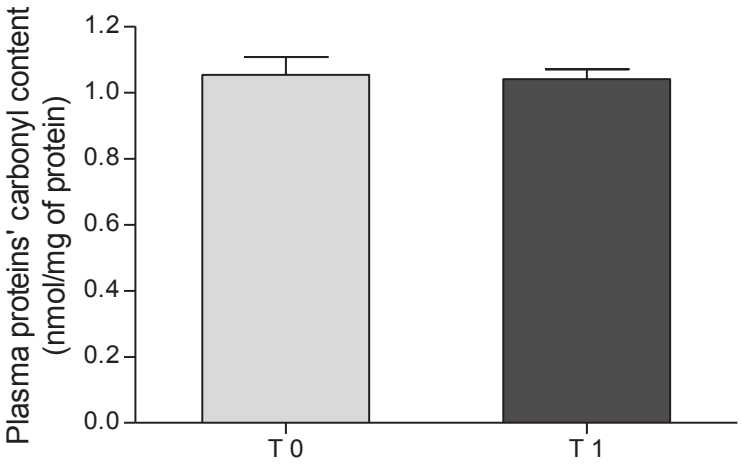

Fig.33. Change in plasma proteins' carbonyl in patients with long-lasting (>16 years) diabetes mellitus (p = 0.817) (values given in nmol/mg protein).

5.4.2. Oxidative stress reactivity during alpha-lipoic acid treatment : plasma proteins' carbonyl and plasma proteins' SH groups– relation with diabetes type

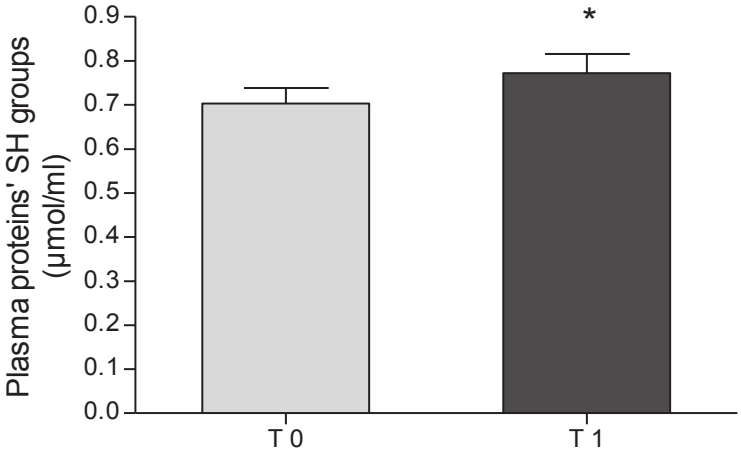

Fig.34. Change in plasma proteins' SH groups in patients with diabetes mellitus type 1 – significant increase (p = 0.037) (values given in µmol/ml of plasma). (*p<0,05)

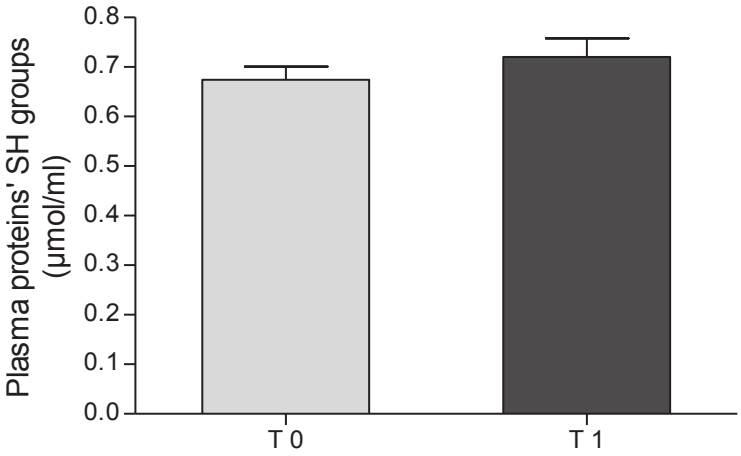

Fig.35. Change in plasma proteins' SH groups in patients with diabetes mellitus type 2 – (p = 0.55) (values given in µmol/ml of plasma).

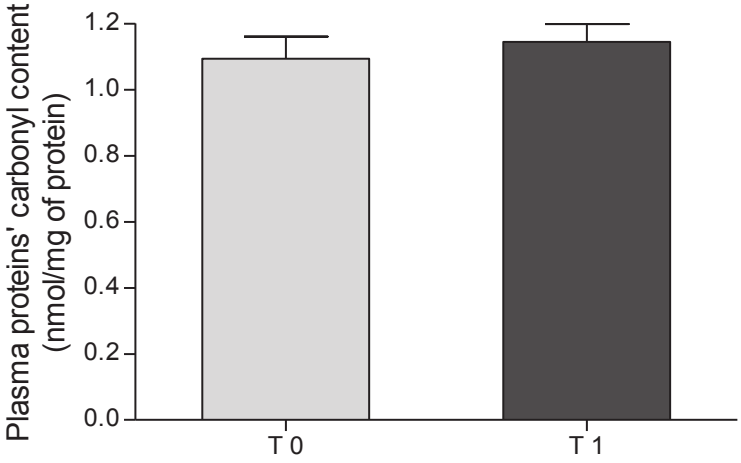

Fig.36. No change in plasma proteins' carbonyl in patients with diabetes mellitus type 1 – (p = 1,00) (values given in nmol/mg protein).

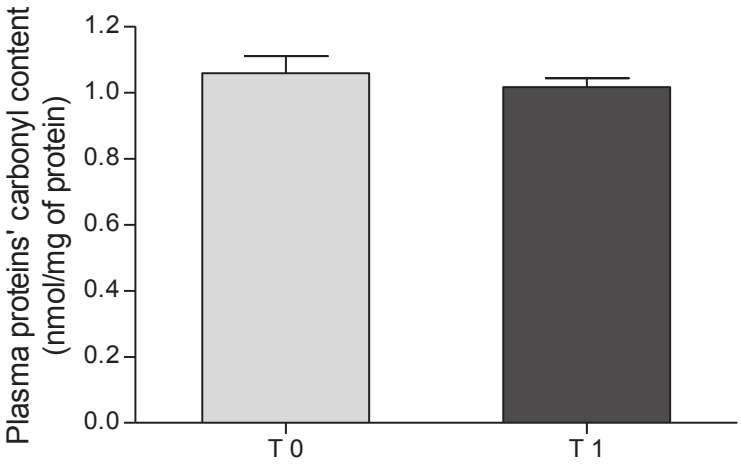

Fig.37. No change in plasma proteins' carbonyl in patients with diabetes mellitus type 2 (p = 0.99) (values given in nmol/mg protein).

5.4.3. Oxidative stress reactivity during alpha-lipoic acid treatment : plasma proteins' carbonyl and plasma proteins' SH groups– relation with calcium blockers treatment

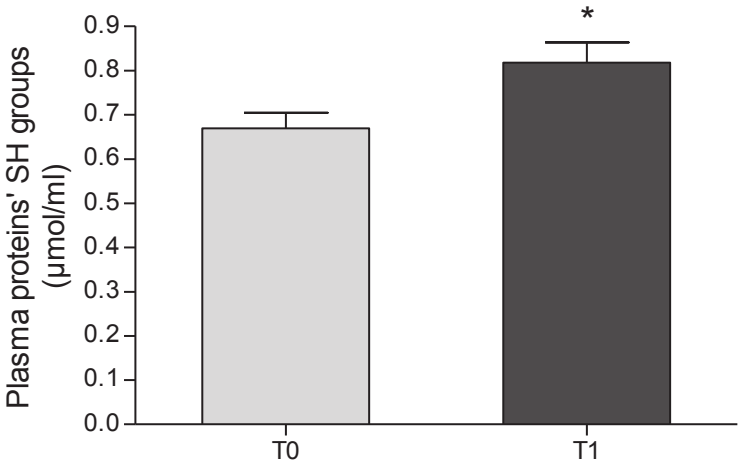

Fig.38. Change in plasma proteins' SH groups in patients with diabetes mellitus type 1 and 2 (type 1 diabetes: 2 patients, type 2 diabetes : 9 patients)-relation with calcium blockers (amlodipine, felodipine, verapamil) treatment (p = 0.03) (values given in µmol/ml of plasma).

Relation with another medicines commonly used (ACE-inhibitors, metformin, nitrates, statins)was not confirmed.

5.5. Questionnaires

5.5.1. VAS – Visual Analog Scale

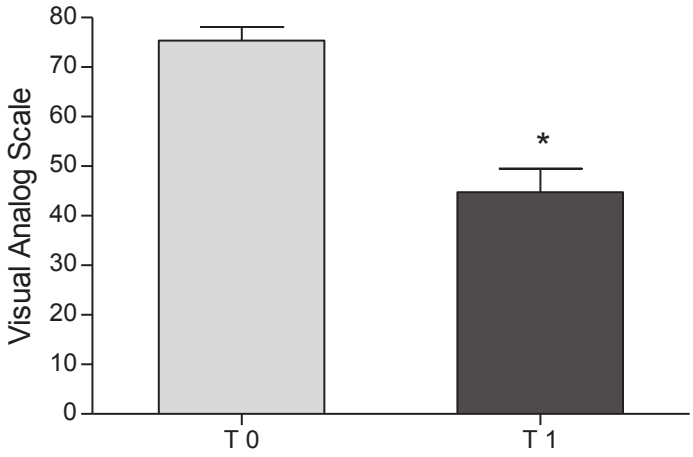

Fig.39. Pain symptoms, described by patients with VAS (Visual Analog Scale) diminished significantly during treatment with alpha-lipoic acid (ANOVA test, $p < 0.0001$) (*$p<0,05$ versus B)

5.5.2. NPS - Neuropathy Pain Score

All answers in Neuropathy Pain Scale reflected diminishing pain. Precise results of Neuropathy Pain Scale answers are given in table below:

Neuropathy Pain Scale 1-2-3 answers	Results of Friedman's ANOVA test (n=27)
1- strength of pain	p < 0,00001
2- sharpness sensation	p = 00015
3- burning sensation	P = 0,0034
4- bruising sensation	P = 0,0007
5- freezing sensation	P = 0,0065
6- sensibility sensation	P = 0,00706
7- itching sensation	P = 0,066
8- focal, reccuring pain	P = 0,18
9- unpleasantness sensation	P = 0,00078
10- deep pain sensation	P = 0,0408
11- superficial pain sensation	P = 0,001

Tab.7. Results of Neuropathy Pain Scale answers – diminishing pain sensation

5.5.3. PQ -Pharmacoeconomy question

Patients noticed improvement regarding pain impact on time spent in work (less and less days out of work), but it was in general not significant (p = 0.219)

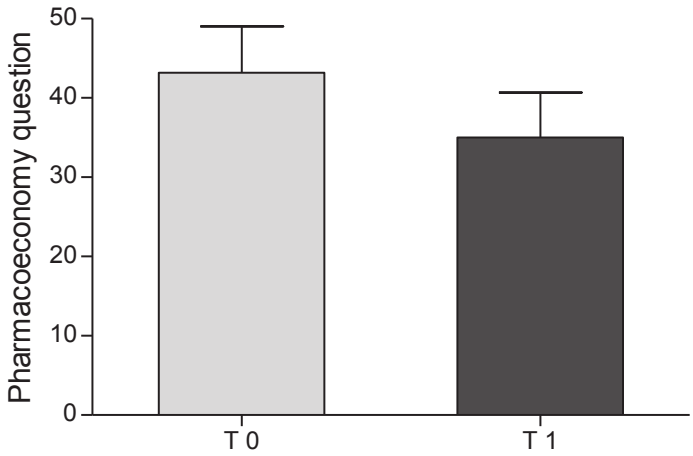

Fig.40. Pharmacoeconomy question results – pain impact on time spent in work – decrease not significant(p = 0.219, ANOVA).

5.5.4. Efficiency of work and vital activity

Patients noticed significant improvement regarding pain impact on everyday efficiency of work and vital activity (PIEV) (p = 0,00229 versus B, ANOVA)

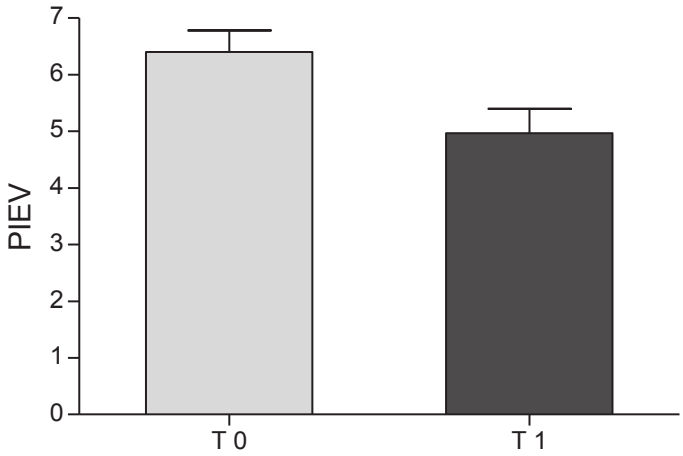

Fig.41. Pain impact on everyday efficiency of work and vital activity (PIEV) (p = 0,00229) (*p<0,05)

5.5.5. Pain, sleep and activity - Likert scale

The significant (p = 0,00008 versus B, ANOVA test) improvement in pain intensity, estimated in the morning using the scale from 1 to 10 (min-max) during last 12 hours before wake-up was achieved along with alpha-lipoic acid treatment.

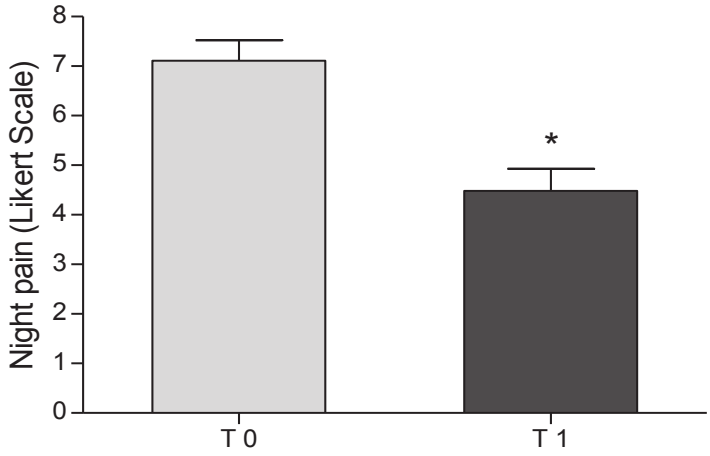

Fig.42. The significant (p = 0,00008) improvement in pain intensity (Likert scale) (*p<0,05 versus B)

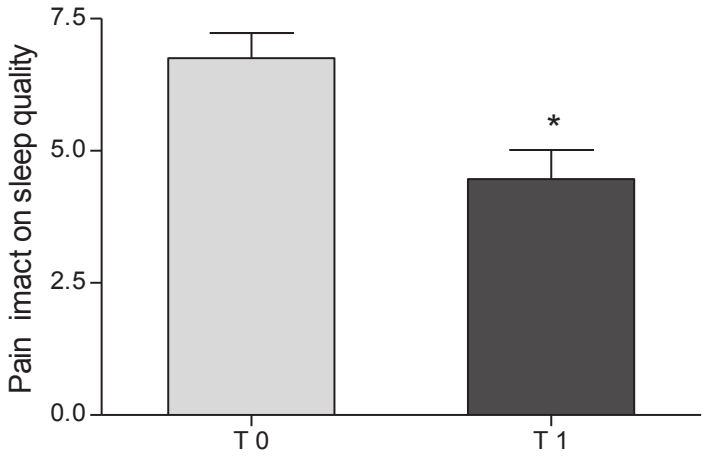

Fig.43. The impact of pain on sleep quality diminished significantly (p = 0,0009). (*p<0,05)

The impact of pain on sleep quality diminished significantly (p = 0,0009). Patients valued the point on the 1-10 (min-max) Likert scale.

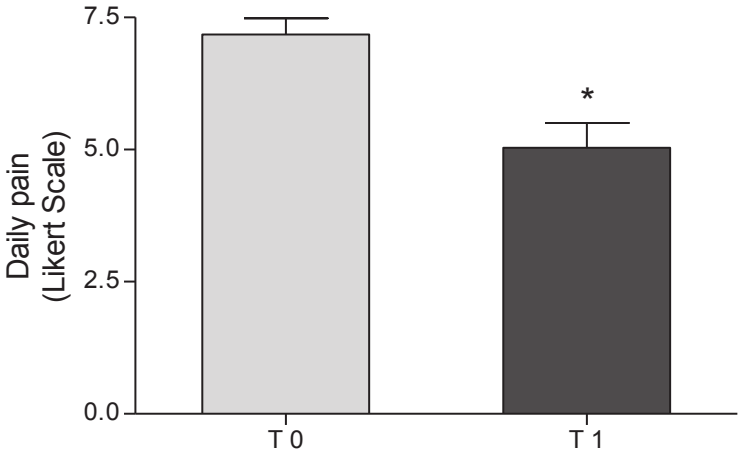

Fig.44. The significant (p = 0,0004) improvement in pain intensity, estimated in the evening (Likert scale) (*p<0,05)

The significant (p = 0,0004) improvement in pain intensity, estimated in the evening using the scale from 1 to 10 (min-max) during last 12 hours of a day was achieved along with alpha-lipoic acid treatment.

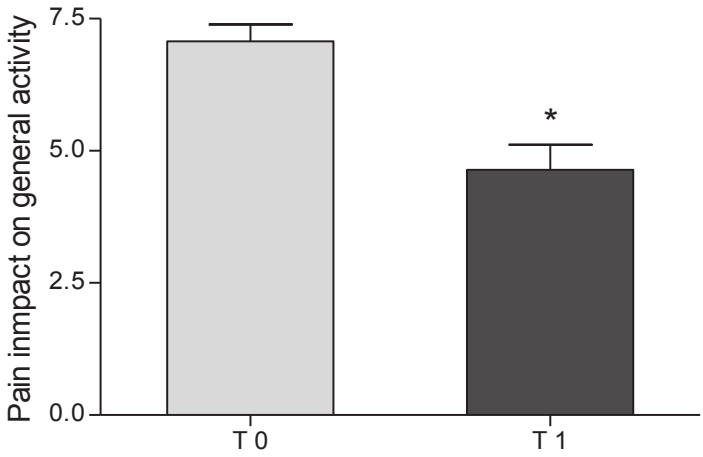

Fig.45. The impact of pain on general activity diminished significantly (p = 0,0001, ANOVA test). (*p<0,05 versus B)

The impact of pain on general activity diminished significantly (p = 0,0001). Patients valued the point on the 1-10 (min-max) Likert scale.

5.5.6. Additional analgesic treatment

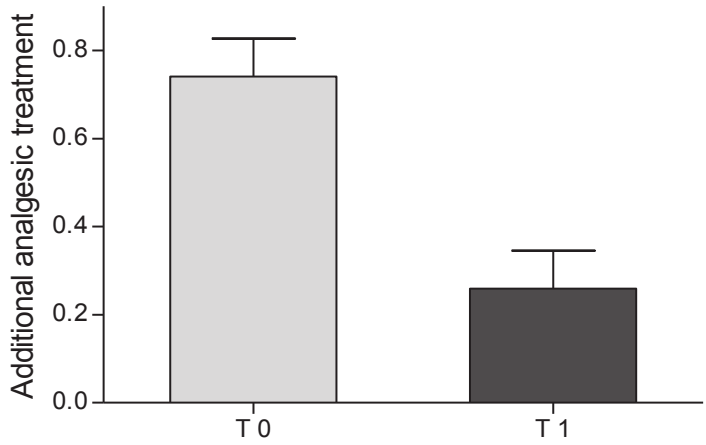

Fig.46. The use of additional analgesic diminished significantly along with the treatment with alpha-lipoic acid. (AAT – additional analgesic treatment)(p=0.000655) (*p<0,05 versus B)

The patients were asked to record the use of additional analgesics throughout the study. The use of additional analgesic diminished significantly along with the treatment with alpha-lipoic acid. (AAT – additional analgesic treatment)(p=0.000655). (*p<0,05 versus B)

5.6. Correlations

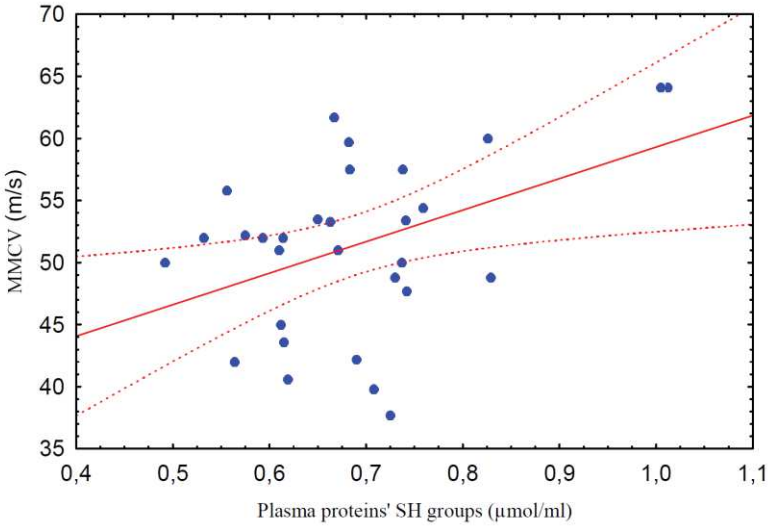

Fig.47. Significant correlation of plasma proteins' SH groups (µmol/ml) with MMCV-median nerve motor conduction velocity (m/s) in the whole studied population of patients with diabetes type 1 and diabetes type 2 (R = 0,43 p<0,05)

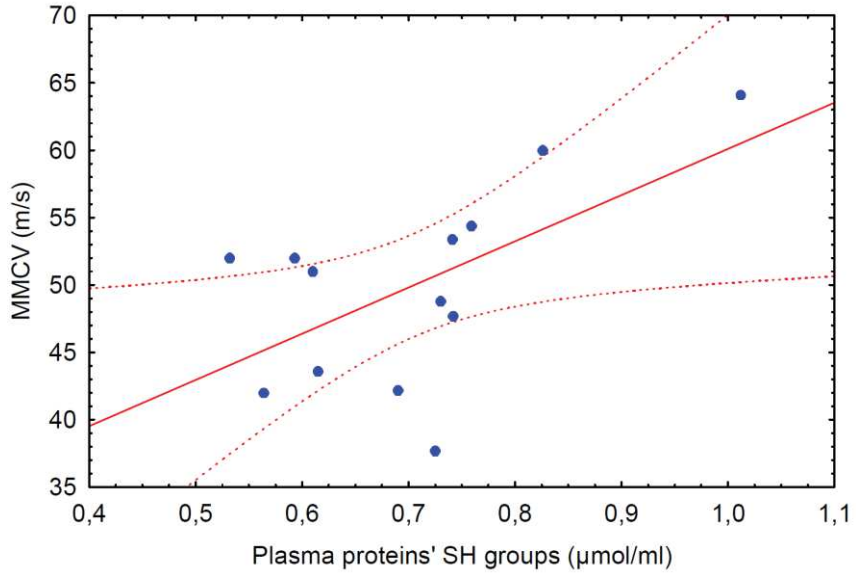

Fig.48. Significant correlation of plasma proteins' SH groups (µmol/ml) with MMCV- median nerve motor conduction velocity (m/s) in the whole studied population of patients with diabetes type 1 (n=13) (R = 0,56 p<0,05)

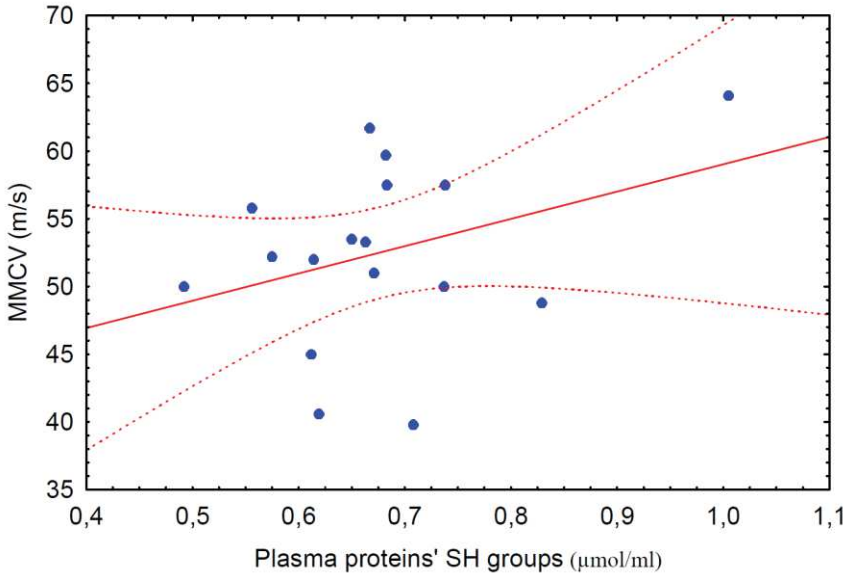

Fig.49. No correlation of plasma proteins' SH groups (µmol/ml) with MMCV- median nerve motor conduction velocity (m/s) in the whole studied population of patients with diabetes type 2 (n=17) (R = 0,34 p>0,05)

Pearson's correlation coefficient was evaluated for a number of parameters: ΔVAS, ΔHbA1c, ΔSH, height, ΔPMCV, ΔMMCV, ΔMNSV, gender, Δcarbonyl. There were no correlation between parameters listed above.

5.7. Ophtalmic assessment

In all patients proliferative retinopathy and other eventual pathologies were excluded. In two patients glaucomatous-like lesions of optic nerve were found, without pathologic intraocular pressure presence.

6. Discussion

6.1 ALA redox effects mechanisms

ALA is used as a drug effective in preventing pain symptoms in diabetic polyneuropathy.

Until recently, the mechanism of analgesic action remains totally unclear. Now it has been demonstrated that the level of pain is influenced by the redox status of the Cav3.2 channel function[51] and the opening/closing of the channel

So far, all bioprotective mechanisms of ALA were attributed to antioxidant activities. Contrary to expectations, lipoic acid proved to be a critical redox-active thiol-channel oxidant resulting in the closure of the channel and reduction of pain sensation.

The results make the interpretations linking the pro- and antioxidant mechanism of action of ALA possible.

Pharmacological doses of ALA enable to achieve therapeutic concentrations in the peripheral nerve by binding to serum albumin. It appears that the ALA firming to the fatty acids binding HAS domain has the opposite effect on the redox state of HSA cysteine34 relative to long chain C16 fatty acids resulting in the Cys34 protection from oxidation and provides a good environment for the cysteine nitrosylation by physiological vasodilator nitric oxide NO. (The role of NO is as a channel Cav3.2)

The consequence of improving the redox state of Cys34 is effective transnitrosylation leading to physiological vasodilatation of vessels supplying the nerves and their electrophysiological function improvement.

It was recently reported that the long chain fatty acids have the opposite effect on albumin-zinc binding[35], which suggests the possibility of ALA albumin function improvement in terms of the fixation of zinc ions of and delivery of this vital ligand to Cav3.2 channels.

[51] Woo Yong Lee, Peihan Orestes, Janelle Latham, Ajit K. Naik, Michael T. Nelson, Iuliia Vitko, Edward Perez-Reyes, Vesna Jevtovic-Todorovic, Slobodan M. Todorovic. Molecular mechanisms of lipoic acid modulation of T-type calcium channels in pain pathway. J Neurosci. 2009 July 29; 29(30): 9500–9509.

The effect of zinc is the channel closure and pain reduction.

New results indicate that oxidation-induced microdomains result in the closing of the ion channel, or limiting the pain sensation, and the oxidation of albumin results in the loss of its biological function. In both molecules, the lipoic acid acts conversely - oxidatively on the channel cysteine, and reductively on the albumin cysteine.

6.2 Neurological parameters

There were positive changes seen in electrophysiological parameters. Neurological parameters measured with electrophysiological tests generally enhanced after treatment with ALA. Improvement was significant in median nerve motor conduction velocity, and median nerve sensory velocity. Both significantly improved parameters regarded shorter nerves, pointing the length of a neuron as an important factor of diabetic pathology and treatment efficacy.

ALADIN II was a croatian 2-year multicenter randomized double-blind placebo-controlled trial establishing long-term response for treatment – 1 week intravenously, afterwards – oral doses. Alpha-lipoic acid appeared to have a beneficial effect on several attributes of nerve conduction in diabetes type 1 and diabetes type 2 patients[52].

SYDNEY trial, performed in Russia[53], measurement of positive sensory symptoms during treatment of diabetic patients with diabetic sensorimotor polyneuropathy with ALA (600 mg infused daily intravenously for 5 days/week for 14 treatments. The primary end point was change of the sum score of daily assessments of severity and duration of TSS. Secondary end points were sum scores of neuropathy signs (NIS), symptoms (NSC), attributes of nerve conduction, quantitative sensation tests (QSTs), and an autonomic test. Intravenous racemic ALA, rapidly and to a significant and meaningful degree, improved such positive neuropathic sensory symptoms as pain and several other neuropathic end points. This improvement of symptoms was attributed to improved nerve pathophysiology, not to increased nerve fiber degeneration.

NATHAN I - multicenter randomized double-masked parallel group clinical trial in North America and Europe regarding 460 diabetic patients with polyneuropathy randomly

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[52] Reljanovic M, Reichel G, et al. Treatment of diabetic polyneuropathy with the antioxidant thioctic acid (alpha-lipoic acid): a two year multicenter randomized double-blind placebo-controlled trial (ALADIN II). Alpha Lipoic Acid in Diabetic Neuropathy. Free Radic Res. 1999 Sep;31(3):171-9.

[53] Ametov AS, Barinov A, Dyck PJ, Hermann R, Kozlova N, Litchy WJ, Low PA, Nehrdich D, Novosadova M, O'Brien PC, Reljanovic M, Samigullin R, Schuette K, Strokov I, Tritschler HJ, Wessel K, Yakhno N, Ziegler D; SYDNEY Trial Study Group.The sensory symptoms of diabetic polyneuropathy are improved with alpha-lipoic acid: the SYDNEY trial. Diabetes Care. 2003 Mar;26(3):770-6.

assigned to oral treatment with α-lipoic acid 600 mg q.d. (*n* = 233) or placebo (*n* = 227) for 4 years. After 4 years, neuropathic deficits progressed significantly on placebo and improved on α-lipoic acid, and the drug was well tolerated throughout the trial[54].

NATHAN II – A long-term multicenter study in North American and Europe - investigates similarily intravenous therapy. The results of the NATHAN II Study have not been published yet.[55]

Oral treatment is investigated in China as well, and also has positive effects with regards to sensory symptoms, but not nerve conduction velocity with ALA dose 600 mg t.i.d. (Chinese trial of 236 diabetics). Individual symptom scores of pain, extremity numbness, burning sensation or resting abnormal sensations were significantly diminished as compared to those before treatment and placebo group (all $P < 0.05$). Nerve conduction velocity had no change. HbA1c further decreased at the end of trial after α-lipoic acid treatment. The major manifestation was burning sensation from throat to stomach (12.7%)[56].

In Romanian work with ALA oral treatment 600 mg once daily similar results were achieved concerning regression of symptoms, nerve conduction velocity improvement and blood glucose lowering[57].

In Russian work describing 27 patients with DM1 short history of DM and mild initial neurological disorders were main predictors of ALA treatment efficacy in diabetic polyneuropathy of the lower limbs[58].

In Chinese trial oral treatment with high-dose α-lipoic acid for 12 weeks may improve symptoms in patients with diabetic polyneuropathy. Dose of 600 mg thrice daily for 2 weeks has marked effects with a reasonable safety, but nerve conduction velocity had no change[59].

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[54] Ziegler D, Low PA, Boulton AJM, Vinik AI, Freeman R, Samigullin R, Tritschler H, Munzel U, Maus J, Schuette K, Dyck PJ. Effect of 4-year antioxidant treatment with α-lipoic acid in diabetic polyneuropathy: the NATHAN 1 trial. Diabetes 2007; 56 (Suppl. 1): A2

[55] Ziegler D, Nowak H, Kempler P, Vargha P, Low PA. Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: a meta-analysis.Diabet Med. 2004 Feb;21(2):114-21.

[56] Gu XM, Zhang SS, Wu JC, Tang ZY, Lu ZQ, Li H, Liu C, Chen L, Ning G. [Efficacy and safety of high-dose α-lipoic acid in the treatment of diabetic polyneuropathy]. Zhonghua Yi Xue Za Zhi. 2010 Sep 21;90(35):2473-6.

[57] Negrişanu G, Roşu M, Bolte B, Lefter D, Dabelea D.Effects of 3-month treatment with the antioxidant alpha-lipoic acid in diabetic peripheral neuropathy. Rom J Intern Med. 1999 Jul-Sep;37(3):297-306.

[58] Bregovskiĭ VB, Posokhina OV, Karpova IA. [Predictors of alpha-lipoic acid treatment efficacy in diabetic polyneuropathy of the lower limbs]. Ter Arkh. 2005;77(10):15-9.

[59] Gu XM, Zhang SS, Wu JC, Tang ZY, Lu ZQ, Li H, Liu C, Chen L, Ning G. [Efficacy and safety of high-dose α-lipoic acid in the treatment of diabetic polyneuropathy]. Zhonghua Yi Xue Za Zhi. 2010 Sep 21;90(35):2473-6.

6.3 Oxidative stress reactivity during alpha-lipoic acid treatment: carbonyl, protein, SH groups

Normal values obtained by different authors for protein carbonyls in mammalian plasma are in range 0,4-1,0 nmol/mg protein[60]. Carbonyl plasma content can increase with age in humans[61], and similarly is higher in older animal brain tissue[62]. There is an age dependent decrease in total thiol groups (T-SH) levels and increase in protein carbonyls (PCO). The levels of plasma oxidized proteins provide an excellent biomarker of oxidative stress due to the relative long half-life of such oxidized proteins.[63]

Initial large dispersion of carbonyl groups (mean 1,073225806, SD ± 0,224742625, range 0,59-1,61; n=32) is evidence of group heterogeneity. Therefore, the usefulness of that parameter is limited. Identical Levin's [64]method was used by Renke[65] when carbonyl groups were determined in pediatric population suffering from juvenile rheumatoid arthiritis (Tab. 9.4.4).

	Children with j.r.a	Healthy children
Carbonyl groups (nmol/mg protein) mean ± SD	1,33±0,67 N=69	0,87±0,18 N=30
Range	0,44-3,49	0,47-1,27

Tab. 9.4.4. Carbonyl groups determined in pediatric population suffering from juvenile rheumatoid arthiritis (j.r.a)[65].

Carbonyl levels after treatment with alpha-lipoic acid are not significantly different.

Carbonyl groups are determined in all serum proteins fractions, mainly in albumins. In the future, serum should be fractionated and eventual changes regarding globulin or albumin oxidation should be observed.

Carbonyl groups determining is a rough estimation of protein oxidation status. In our group of patients this is better visible when SH groups are determined. SH can be good parameter for diabetic patients evaluation.

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[60] Halliwell B., Gutteridge J. Free Radicals In Biology and Medicine. Oxford University Press, 1999, p.417.

[61] Ü.Mutlu-TürkoImage lu, E.lhan, S.Öztezcana, A.Kuru, G.Aykaç-Toker,M.Uysala Age-related increases in plasma malondialdehyde and protein carbonyl levels and lymphocyte DNA damage in elderly subjects Clinical Biochemistry, Volume 36, Issue 5, July 2003, Pages 397-400

[62] Head E, Liu J, Hagen TM, Muggenburg BA, Milgram NW, Ames BN, Cotman CW. Oxidative damage increases with age in a canine model of human brain aging.J Neurochem. 2002 Jul;82(2):375-81.

[63] K. Bhooshan Pandey, M. Murtaza Mehdi, P. Kumar Maurya, S. Ibrahim Rizvi. Plasma protein oxidation and its correlation with antioxidant potential during human aging. Disease Markers, Vol. 29, Nr 1 / 2010; 31-36

[64] Levine R.L., Garland D., Oliver C.N., Amici A., Climent I., Lenz A.G., Ahn B., Shaltiel S., Stadtman E.R.: Determination of Carbonyl Content In Oxidatively Modified Proteins, Meth. Enzymol. 1990, 186, 464-478.

[65] Renke J. Wolnorodnikowe uszkodzenia białek surowicy oraz bariera antyoksydacyjna organizmu w przebiegu młodzieńczego przewlekłego zapalenia stawów. Doctoral thesis Gdańsk 2000. (Abstract In english)

SH groups oxidation take place only in cysteine 34 – the only one cysteine in human albumin.

SH groups levels increase 1 month after ALA intravenous treatment – it means, that neuronal function improvement depends on albumin cystein34 SH groups.

Attempts to assess the thiol status of albumin are based almost exclusively on the isolation of albumin from plasma[66],[67], platelet rich plasma[68] and assessment of redox status of Cys34 after albumin isolation. These methods do not prevent rapid oxidation of albumin, that lowers the results of SH-groups. Some works give low weird values, that are an artifact and mistaken evidence of not present severe illness. Moreover, research conducted in the Peter J.Sadler[69] laboratory proves that it is a source of methodical error associated with the fact that the human serum albumin is redox stable in plasma, but it is immediately oxidized upon isolation (Fig.5.). On the other hand, the different centrifuge setup, not always described, results in big discrepancies.

The only known to us work[70] , where the determination of SH groups performed similar method in participants whose age corresponded with our patients - average age of study participants was 34.85, can be a reference to our results.

Markings were performed on serum albumin, which redox status was most similar to the actual in vivo conditions, not the isolated albumin, which quickly oxidizes.

Results of Wozniak[71] achieved with identical methods – but cannot be considered as control values, because they are much higher and carried out at a much younger age group . However, the results indicates a correlation of albumin SH groups oxidation with age.With proper experiment execution plasma thiols amount will be equal to the amount of albumin[72] ; whereas albumin SH-groups after the isolation are mistakenly lowered due to oxidation depending on the conditions to 50-70%[73]

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[66] Kawakami A1, Kubota K, Yamada N, Tagami U, Takehana K, Sonaka I, Suzuki E, Hirayama K.Identification and characterization of oxidized human serum albumin. A slight structural change impairs its ligand-binding and antioxidant functions. FEBS J. 2006 Jul;273(14):3346-57.

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7.1. Biochemical parameters influenced by alpha-lipoic treatment

7.1.1. HbA1c

HbA1c after treatment with alpha-lipoic acid in whole group decreased significantly ($p = 0,043$). In the type 1 diabetes group, HbA1c decrease was not significant ($p = 0,084$), whereas in the type 2 diabetes group, HbA1c decrease was significant ($p = 0,017$). Alpha-lipoic acid influences insulin sensitivity, and non-insulin dependent glucose uptake, and that is one reason of these differences. On the other hand, type 1 diabetes patients were educated in use of functional insulin therapy, free in adjusting doses, and being more prone to hipoglycaemia they could reduce doses of insulin trying to achieve stable glucose levels.

Similar work was done by Huang EA, Gitelman SE, who used an oral controlled-release formulation of alpha-lipoic acid (CRLA). In that article 40 type 1 diabetes patients were studied. There was no significant change in any measurement of oxidative damage, total antioxidant status, HbA1c, or microalbuminuria prevalence after treatment with either placebo or CRLA[74].

In another paper seventy-one children and adolescents with type 1 diabetes, using intensified insulin therapy, were treated with oral alpha-lipoic acid. The analyses showed no differences at baseline and after 3 and 6 months among the three groups: None of BMI, 24 h blood pressure, lipid profile, HbA1c, dietary habits, and body composition resulted in any differences between the three groups or over the study period[75].

In another article, ALA improves glucose effectiveness and lowers fasting glycaemia in lean and obese type 2 diabetic patients[76].

ALA, a natural cofactor of mitochondrial dehydrogenase complexes and a potent antioxidant, improves glucose metabolism in people with type 2 diabetes mellitus and in animal models of diabetes. There is some evidence, that that R (+) alpha-lipoic acid directly activates tyrosine and serine/threonine kinases in target cells, which could lead to the stimulation of glucose uptake induced by this natural cofactor. These properties are unique among all agents currently used to lower glycaemia in animals and humans with diabetes[77]. Mechanism regarding GLUT4 receptor muscle content increasing and reducing glycemia in streptozotocin-diabetic rats is described elsewhere by Israel investigators[78].

HbA1c is a known classical risk factor that predicts the development of diabetic neuropathy[79].

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7.1.2. Blood count

No significant changes in the blood count during the use of alpha-lipoic acid were noted. There is no information about such changes in the literature.

7.1.3. Blood chemistry

Erythrocyte sedimentation rate, creatinine level, Aspat, Alat were not changed. Falk, GGTP, HDL and triglicerydes did not change. Bilirubin level, total cholesterol and LDL did not change.

7.1.4. Microalbuminuria

In presented study microalbuminuria did not change.

7.2. Oxidative stress reactivity during alpha-lipoic acid treatment : carbonyl, SH groups – relations with diabetes duration, diabetes type, concomittant treatment

7.2.1. Oxidative stress reactivity during alpha-lipoic acid treatment : carbonyl, SH groups – relation with diabetes duration

Carbonyl levels and SH groups after treatment with alpha-lipoic acid regarding both shorter and longer diabetes duration are not significantly different.

7.2.2. Oxidative stress reactivity during alpha-lipoic acid treatment : carbonyl, SH groups – relation with diabetes type

Carbonyl levels after treatment with alpha-lipoic acid regarding both diabetes type 1 and diabetes type 2 are not significantly different. However, there was a significant increase on SH groups in diabetes type 1 patients ($p = 0.037$). Probably the free fatty acids metabolism is the difference between types 1 and 2 cause.

7.2.3. Oxidative stress reactivity during alpha-lipoic treatment – influence of concomittant treatment

As the project regarded patients treated under standard conditions, most of them were receiving beside insulin, another different drugs. These included gliclazide, angiotensin converting enzyme inhibitors, calcium blockers and statins (or HMG-CoA reductase inhibitors). Some listed medicines diminish oxidative stress, hence influence

somehow carbonyl protein groups determination[80,81,82,83,84,85,]. Many patients, who underwent ALA treatment, were receiving these drugs : 3 from 31 were treated with gliclazide, 16 out of 31 were treated with HMG-CoA reductase inhibitors : simvastatin or atorvastatin, 22 out of 31 were treated with ACE-inhibitors.

There was significant positive change in plasma proteins' SH groups observed in patients with diabetes mellitus type 1 and 2 (type 1 diabetes: 2 patients, type 2 diabetes - 9 patients) in relation to calcium blockers (amlodipine, felodipine, verapamil) treatment ($p = 0.03$).

Until now, there were no experimental evidence in in vivo models regarding calcium chanel blockers antioxidant activity[86]

Relation with another medicines commonly used (ACE-inhibitors, metformin, nitrates, statins) was not confirmed.

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7.3. Questionnaires

7.3.1. VAS – Visual Analog Scale

VAS - is a measurement tool for subjective pain feeling, of continuous (or "analogue") aspect.
The VAS can be compared to other linear scales such as the Likert scale or Borg scale. The sensitivity and reproducibility of the results are broadly very similar, although the VAS may outperform the other scales in some cases[87].

VAS can be presented in different ways, including vertical lines and lines with extra descriptors. The benefits and shortcomings of different styles of VAS are informatively discussed by Wewers & Lowe (1990)[88].
As such an assessment is course highly subjective, the scales are of most value when looking at change within individuals, and are of less value for comparing across a group of individuals at one time point. It could be argued that the scale is trying to produce interval/ratio data out of subjective values that are at best ordinal. So, some caution is required in analizing the data. Some researchers prefer to use another method that is based on the rank ordering of scores rather than their exact values, to avoid reading too much into the precise VAS score.
Observing the changes over time gives practical clear insight of patients pain symptoms.

Every patient of studied population was asked to match his own level of pain on VAS scale three times during the study : at the beginning, soon after 15 days of therapy with alpha – lipoic acid, and a month later.

Alpha-lipoic acid administered intravenously was strongly effective in diminishing pain symptoms (ANOVA test, $p < 0{,}0001$) in patients with painful diabetic neuropathy, what was assessed with the VAS scale.

First trial valuating pain symptoms relief after ALA treatment was ALADIN I – german 3-week multicentre controlled trial[89] - based on intravenous ALA treatment comparing

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three doses – 1200, 600 and 300 mg. Treated population was 328 non-insulin dependent diabetic patients with neuropathy. Findings substantiated that intravenous treatment with alpha-lipoic acid using a dose of 600 mg/day over 3 weeks was superior to placebo in reducing symptoms of diabetic peripheral neuropathy, without causing significant adverse reactions.

ALADIN III was a german 7-month multicenter randomized controlled trial[90] ranging 509 diabetes type 2 patients treated 3 weeks intravenously, followed by 6 months oral treatment with alpha-lipoic acid. Eventually, Total Symptom Score (TSS) for neuropathic symptoms (pain, burning, paresthesias, and numbness) in the feet, and the Neuropathy Impairment Score (NIS), which both decreased just after i.v. treatment was done.

ORPIL was a small german oral pilot study for establishing the efficacy and dose response of oral treatment with 600 mg ALA t.i.d on neuropathic symptoms and deficits in type 2 diabetic patients with symptomatic DSP [91].

SYDNEY II – trial performed in Russia and Israel; 181 diabetic patients with neuropathy were treated with oral doses of 600 mg a day (n = 45), 1,200 mg (n = 47), and 1,800 mg of ALA (n = 46) or placebo (n = 43) for 5 weeks. The primary end point was the change from baseline of the TSS (Total Symptom Score), describing stabbing pain, burning pain, paresthesia, and asleep numbness of the feet. Secondary outcome measures included individual grading of TSS, Neuropathy Symptoms and Change (NSC) score, Neuropathy Impairment Score (NIS), and patients' overall assessment of efficacy. Safety analysis showed a dose-dependent increase in nausea, vomiting, and vertigo. Oral treatment with ALA for 5 weeks improved neuropathic symptoms and deficits in patients with distal symmetric polyneuropathy. An oral dose of 600 mg once daily appears to provide the optimum risk-to-benefit ratio. [92]

7.3.2. NPS - Neuropathy Pain Score

All answers in Neuropathy Pain Scale reflected pain diminishing.

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7.3.3. PF -Pharmacoeconomy question and Efficiency of work and vital activity

Patients noticed improvement regarding pain impact on time spent in work (less and less days out of work), but it was in general not significant (p = 0,16530, ANOVA). Most of the patients are pensioners, so they do not work actively, but have some activity – taking care of their homes and children.

7.3.4. Pain, sleep and activity - Likert scale

The significant (p = 0,00008, ANOVA test) improvement in pain intensity, estimated in the morning using the scale from 1 to 10 (min-max) during last 12 hours before wake-up was achieved along with alpha-lipoic acid treatment. The impact of pain on sleep quality diminished significantly (p = 0,0009 , ANOVA test). Patients valued the point on the 1-10 (min-max) Likert scale. The significant (p = 0,0004, ANOVA test) improvement in pain intensity, estimated in the evening using the scale from 1 to 10 (min-max) during last 12 hours of a day was achieved along with alpha-lipoic acid treatment. The impact of pain on general activity diminished significantly (p = 0,0001, ANOVA test). Patients valued the point on the 1-10 (min-max) Likert scale.

7.3.5. Additional analgesic treatment

The patients were asked to record the use of additional analgesics throughout the study. The use of additional analgesic diminished significantly along with the treatment with alpha-lipoic acid. (AAT – additional analgesic treatment)(p=0,00655)

7.4. Correlations

Pearson's correlation coefficient was performed for a number of parameters: ΔVAS, ΔHbA1c, ΔSH, height, ΔPMCV, ΔMMCV, ΔMNSV, gender, Δcarbonyl.

There were no correlation observed between these parameters; especially, between ΔMNSV correlated with ΔSH – Pearson's correlation coefficient was equal to 0,6 (=0,025).

Histidine 191[93] of $Ca_v3.2$ channel is important for the modulation of the redox agents. It is also a critical element in the binding of the zinc ion in the channel and appears to be important for binding and supply of the zinc ion by albumin molecules (it is suggested[94] that the binding more than two fatty acid molecules to the albumin decreases the binding capacity of zinc, i.e. reduces the supply the main inhibitor of the $Ca_v3.2$.channel)

Cysteine34 redox status evaluation upon the lipoic acid delivery may provide indirect information relating to the supply of zinc to the $Ca_v3.2$ channel sensory neurons or the presence of ALA in the vascular bed – bound to albumin – that restores impaired albumin ability to bind zinc.

In diabetes, as a result of prolonged exposure of albumin to elevated level of fatty acids in the plasma there are limitations of the albumin-specific binding of zinc, what can amplify diabetic neuropathy and depression[95].

[93] Kang HW1, Vitko I, Lee SS, Perez-Reyes E, Lee JH. Structural determinants of the high affinity extracellular zinc binding site on Cav3.2 T-type calcium channels.J Biol Chem. 2010 Jan 29;285(5):3271-81.

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8. Conclusions

Set work goals have been achieved.

The effect of treatment with alpha-lipoic acid on selected parameters of redox state of plasma protein (80% of which is albumin) in patients with diabetic neuropathic hyperalgesia has been defined. It allows for a deeper insight into the neuropathy patomechanism elements.

The beneficial effect of treatment to improve nerve conduction in terms of median nerve motor conduction velocity and median nerve sensory velocity was confirmed. Both significantly improved parameters regarded shorter nerves, pointing the length of a neuron as an important factor of diabetic pathology and treatment efficacy.

The findings indicate that a 15-days intravenous treatment with racemic alpha-lipoic acid (600 mg/day) had a clinically meaningful effect on neuropathic symptoms and signs, especially associated with favourable effect on neuropathic deficits without causing significant adverse reactions. The improvement of symptoms is attributed to improved nerve pathophysiology.

Conduction velocity in the hands and legs improved significantly as a result of treatment . Changes in other conduction parametres, despite the lack of statistical significance indicate a strong neurotropic treatment potential.

As a result of the treatment the nerve conduction can improve, indicating that the pathology may be although partially reversible and clinically controlled[96],[97].

Intravenous administration of alpha lipoic acid was associated with a decreased HbA1c, and within a subgroup of patients with type 2 diabetes the reduction was statistically significant.

Carbonyl levels after treatment with alpha-lipoic acid are not significantly different.

Lipoic acid treatment results in a reduction of oxidized albumin, but does not protect it against carbonylation. Tthe carbonylation is caused by the transition metals activity, eg. iron. It seems that lipoic acid is not significantly effective as a biological transition metal chelator in vitro in this group of patients.

Studies in vitro (unpublished) performed on human albumin treated with isolated iron ions have shown that alpha-lipoic acid is much weaker than iron-chelator bioflavonoids.

Interesting and encouraging for future speculations and research were results of serum SH groups measurements. Long-term effect is possibly connected with serum albumin SH groups.

SH groups oxidation take place only in cysteine 34 – the only one free cysteine in human albumin.

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SH groups levels increase 1 month after ALA intravenous treatment – it means, that neuronal function improvement depends on albumin cystein34 SH groups.

We did not evaluate the status of isolated albumin because the process of isolation involves the irreversible oxidation of albumin in a short time. Serum albumin storaged as plasma solution maintains properties for a long time.

A very small group of patients receiving calcium channel blockers received a much better improvement in the status of the thiol of albumin (see Limitations of the work).

One can wonder whether monitoring of SH groups could serve as a diagnostic test and evaluation of lipoic acid therapy.

Diabetes mellitus is a free radical – associated disease. The use of certain antioxidants may contribute to improvement of prognosis and regression of late complications of diabetes.

The treatment with ALA, focused on mitochondria metabolism and neuronal status may, in some years time, become central to symptomatic painful diabetic neuropathy trials.

Medications satisfy the criterion of efficacy on the metabolic pathways involved in the development of diabetic complications are the hope for the improvement of prognosis in patients with diabetes.

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10. Summary

The adult diabetic patients were recruited from APR2005 to NOV2006 from population of Regional Diabetes Center of Gdansk Medical University, Poland. Research took place with the approval of the Local Ethics Comitee. Informed consent was obtained from all subjects eligible to participate in the study. Inclusion criteria were type 1 or type 2 diabetes according to World Health Organisation/American Diabetes Asociation/Polish Diabetes Association criteria, age 20-70 years, clinically evident polyneuropathy, insulin treatment. Exclusion criteria were: 1) non-diabetic neuropathy; 2) smoking or non-smoking<1 year; 3) use of antioxidants (vitamin C, E, probucol, β-karotene, carvedilol, iron chelators, α-lipoic acid) or prooxidants (iron) within the last 3 months; 4) peripheral arterial disease (signs or symptoms); 5) coronary heart disease, myocardial infarction, heart failure, cardiac peacemarker; 6) any medication affecting neurological functions; 7) neurological diseases; 8) blood glucose levels>400mg/dl and/or ketonuria; 9) alcohol abuse; 10) proliferative retinopathy; 11) any systemic insufficiency; 12) any serious disease or instability.

The effect of treatment with alpha-lipoic aced was studied prospectively in 31 patients with diabetes. All patients received alpha-lipoic acid (Thiogamma, Wörwag) intravenously at a dose of 600 mg daily for 15 days.

Parameters of oxidative stress were examined - SH group concentration and the concentration of carbonyl serum albumin. At the same time the nerve conduction studies and the assessment of pain sensation were performed.

Motor and sensory conduction velocities, vibration sensation thresholds, Hba1c, Plasma thiols and carbonyl groups were measured at baseline, after 15 days of treatment and 15 days after the end of treatment.

A lot of nerve conduction studies parametres were enhanced, and median nerve motor conduction velocity improved significantly.

The decrease of Hba1c in the whole studied population with diabetes type 1 and diabetes type 2 and painful neuropathy was observed.

In the type 1 diabetes group (n = 13), HbA1c decrease was not significant (p = 0,067). (T0-before treatment, T1-after 15 days of treatment with intravenous 600 mg alpha-lipoic acid). Lack of significancy is probably due to insulin dose adjustements, more easy to achieve in good educated diabetes type 1 patients.

In the type 2 diabetes group (n = 17), HbA1c decrease was significant (p = 0,0074). (T0-before treatment, T1-after 15 days of treatment with intravenous alpha-lipoic acid). The effect was significant probably due to stable insulin dosing.

Change in plasma proteins' SH groups was observed, especially in patients with diabetes mellitus type 1 – was significantly higher comparing to baseline(*p<0,05).

No change in plasma protein after treatment with alpha-lipoic acid was observed.

Pain symptoms, described by patients with VAS (Visual Analog Scale) (ANOVA test, p < 0.0001) (*p<0,05 versus B).and Likert scale (*p<0,05) diminished significantly during treatment with alpha-lipoic acid

The results indicate a selective beneficial effect of alpha-lipoic acid on the redox state of serum albumin. Due to the fact that albumin cystein34 is the main carrier of nitric oxide, it is hypothesized that the cysteine thiol protection may be an important part of the neuroprotective action of the lipoic acid.

11. Abstract

Title

Evaluation of selected etiopathogenic and clinical painful diabetic neuropathy during treatment with alpha-lipoic acid administered intravenously at a dose of 600 mg per daily.

Summary

The study involved adult patients with painful diabetic neuropathy. Parameters of oxidative stress were examined - SH group concentration and the concentration of carbonyl serum albumin.

At the same time the nerve conduction studies and the assessment of pain sensation were performed

The results indicate a selective beneficial effect of alpha-lipoic acid on the redox state of serum albumin. Due to the fact that albumin cystein34 is the main carrier of nitric oxide, it is hypothesized that the cysteine thiol protection may be an important part of the neuroprotective action of the lipoic acid.

12. Streszczenie w języku polskim

Tytuł

Ocena wybranych parametrów klinicznych i etiopatologicznych bolesnej neuropatii cukrzycowej podczas leczenia za pomocą kwasu alfa-liponowego stosowanego dożylnie w dawce 600 mg dziennie.

Streszczenie

Badaniu poddano grupę dorosłych pacjentów z bolesną neuropatią cukrzycową. Badano parametry stresu oksydacyjnego - stężenie grup SH oraz stężenie karbonyli albuminy osocza. Jednocześnie wykonywano badania przewodnictwa nerwowego. Uzyskane wyniki wskazują na selektywnie korzystny wpływ kwasu alfa-liponowego na stan oksydoredukcyjny albuminy osocza. Z uwagi na to, że cysteina34 albuminy jest głównym nośnikiem tlenku azotu, postawiono hipotezę, że ochrona tiolu cysteiny może być istotnym elementem neuroprotekcyjnego działania kwasu liponowego.

13. List of Tables

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16. Rozwinięte streszczenie w języku polskim

Tytuł

Ocena wybranych parametrów klinicznych i etiopatologicznych bolesnej neuropatii cukrzycowej podczas leczenia za pomocą kwasu alfa-liponowego stosowanego dożylnie w dawce 600 mg dziennie.

Praca doktorska

Promotor: Prof.dr hab. Michał Woźniak Katedra Chemii Medycznej Gdańskiego Uniwersytetu Medycznego

Gdańsk, 2016

Projekt został wykonany po uzyskaniu zgody Komisji Bioetycznej w Gdańsku

Streszczenie

Badaniu poddano grupę dorosłych pacjentów z bolesną neuropatią cukrzycową. Badano parametry stresu oksydacyjnego - stężenie grup SH oraz stężenie karbonyli albuminy osocza. Jednocześnie wykonywano badania przewodnictwa nerwowego. Uzyskane wyniki wskazują na selektywnie korzystny wpływ kwasu alfa-liponowego na stan oksydoredukcyjny albuminy osocza. Z uwagi na to, że cysteina34 albuminy jest głównym nośnikiem tlenku azotu, postawiono hipotezę, że ochrona tiolu cysteiny może być istotnym elementem neuroprotekcyjnego działania kwasu liponowego.

WSTĘP

Przewlekła hiperglikemia charakterystyczna dla cukrzycy i związane z nią zaburzenia metaboliczne prowadzą do rozwoju przewlekłych powikłań cukrzycy, a te z kolei do zwiększonej zachorowalności i śmiertelności w tej chorobie.

Praca poświęcona jest chemii medycznej neuropatii cukrzycowej, w kontekście leczenia pacjentów z neuropatią bólową obwodową kwasem alfa-liponowym.

Niezależnie od typu cukrzycy u podłoża rozwoju powikłań leży zaburzenie procesów oksydoredukcyjnych na poziomie komórki i całego organizmu.

Glutation jako kluczowy czynnik redukcyjny okazuje się mieć niewielkie znaczenie dla ochrony neuronów z uwagi na jego niskie stężenie w tkance nerwowej.

Dużą rolę w ochronie neuronalnych układów redoksowych, ale również w regulacji komórkowej odpowiedzi na insulinę okazują się mieć grupy tiolowe, np. fosfataz tyrozynowych. Jedna z nich, PTP1B, w neuropatii cukrzycowej wykazuje zwiększoną aktywność.

Nadtlenek wodoru - H_2O_2. generowany w śródbłonku przy udziale oksydazy NADPH (izoformy NOX_4) jest naturalnym inhibitorem PTP1B. Być może na tej drodze NOX-4 bierze udział w komórkowej odpowiedzi na insulinę.

Kwas alfa-liponowy, lek obecnie stosowany w leczeniu neuropatii cukrzycowej, jest inhibitorem śródbłonkowej oksydazy NADPH, przez co poprawia funkcję endothelium.

Albumina ludzka wydaje się być głównym nośnikiem tiolanowych reszt redukcyjnych w surowicy, czyli głównym antyoksydantem. 34 cysteina albuminy stanowi 95% grup SH osocza. U osób z cukrzycą nasilony stres oksydacyjny i bezpośrednio – wiązanie ponad dwóch reszt wolnych kwasów tłuszczowych – indukuje poprzez zmianę konformacji utlenienie tiolanu 34 cysteiny. Utleniona cząsteczka albuminy traci swoje własności antyoksydacyjne oraz możliwość wiązania tlenku azotu, pełniącego rolę fizjologicznego wazodylatatora. To prowadzi z kolei do uszkodzenia mikrokrążenia nerwów obwodowych, co leży u podłoża neuropatii cukrzycowej.

Nadtlenek wodoru prowadzi do utlenienia albuminy, a w reakcjach z metalami przejściowymi – żelazem, miedzią – generuje powstawanie bardzo toksycznego rodnika ponadtlenkowego, który uszkadza boczne łańcuchy albuminy generując powstawanie grup karbonylowych w albuminie.

Status tiolowy albuminy badany był dotychczas prawie wyłącznie na albuminie izolowanej z osocza. Niestety proces iziolacji albuminy prowadzi do błyuskawicznego jej utleniania, co zaniża wyniki oznaczania grup SH. Dlatego część publikacji zawiera dziwne wyniki, które mają świadczyć o zaburzeniu redoksowym, a są artefaktem. Obecna praca bada status tiolowy albuminy *in vivo*.

Oksydacja kluczowych białek organizmu człowieka jest jednym z głównych molekularnych biomarkerów polineuropatii. Te białka to przede wszystkim albumina osocza, ale także białka receptorów, kanałów np. wapniowych, zależnych od potencjału, zlokalizowanych w neuronach sensorycznych i komórkach śródbłonka *vasa nervorum*.

Kanałem jonowym regulowanym procesami redox jest receptor neuronu czuciowego Cav3.2T . Redukcja tiolanu receptora prowadzi do otwarcia kanału i napływu jonów wapniowych, co prowadzi do polaryzacji błony komórkowej i powstania uczucia bólu.

Kwas alfa-liponowy utlenia reszty tiolowe receptora i odwraca opisany efekt. Świadczyć to może o fakcie prooksydacyjnego działania kwasu liponowego.

Przeciwstawnie na białka receptora działają jony metali przejściowych (żelaza, miedzi).

Zjawiska redoksowe mają wpływ także na funkcję innych receptorów, takich jak kanały TRP i kanały wapniowe typu T. Występują one w neuronach czuciowych i ruchowych oraz w komórkach śródbłonka. Ich funkcja bezpośrednio zalezy od wysycenia albuminy tlenkiem azotu. Kwas alfa-liponowy wykazuje silny wpływ na ich stan redoksowy i czynność, modulując ból neuropatyczny.

Innym czynnikiem mającym ewidentny wpływ na funkcję białek u osób z cukrzycą jest glikacja. Glikowana albumina może przyłączac więcej jonów metali przejściowych i z tego powodu ulegać silniejszemu utlenieniu. Podobnie jest w przypadku kanału

wapniowego Cav3.2 w błonie neuronów czuciowych – glikacja reszt asparaginy prowadzi do otwarcia kanału i hiperalgesii i allodynii. Deglikacja odwraca ten efekt.

Tlenek azotu jest efektywnym elektrofilem biorącym udział w S-nitrozylacji reszt cysteiny kanału Cav3.2 T-type , co prowadzi do zmniejszenia czucia bólu w neuropatii. Biodostępność tej cząsteczki jest jednak niska z uwagi na krótki czas półtrwania oraz upośledzenie funkcji syntazy tlenku azotu u osób z cukrzycą.

Cysteina 34 albuminy ludzkiej jest sensorem tlenku azotu i po nitrozylacji staje się głównym źródłem tlenku azotu aktywnego zarówno w wazodylatacji jak i innych działaniach receptorowych.

W warunkach typowych dla cukrzycy i zespołu metabolicznego cząsteczka albuminy przyłącza ponad dwie reszty kwasów tłuszczowych co prowadzi do izomeryzacji cis-trans prolina-cysteina 34, translokacji cysteiny 34 z wnętrza kieszonki N-końcowego łańcucha albuminy na powierzchnię białka i w konsekwencji jej utlenienie. Ta redoksowa zmiana cysteiny 34 zmniejsza zdolność wiązania tlenku azotu przez albuminę.

W efekcie biodostępność tlenku azotu spada a objawy neuropatii nasilają się.

Kolejnym czynnikiem ograniczającym biodostępność tlenku azotu jest nadekspresja i aktywacja oksydazy NADPH w komórce śródbłonka. Enzym ten generuje powstawanie anionorodnika ponadtlenkowego, który inaktywuje tlenek azotu.

Kwas alfa-liponowy jest jednym z inhibitorów oksydazy NADPH .

CEL PRACY

Celem pracy jest odpowiedź na pytanie, czy kwas alfa-liponowy ma wpływ na status cysteiny 34 albuminy ludzkiej oraz poziom utlenowania łańcuchów bocznych aminokwasów poprzez oznaczenie stężeń grup karbonylowych, który może mieć wpływ na fizjologiczną funkcję albuminy.

Albumina jest głównym źródłem tlenku azotu i jonów Zn^{2+}, inhibitorów kanału Ca$_v$3.2 , stąd też powstaje pytanie, czy poprawa jej funkcji biologicznej po zastosowaniu kwasu alfa-liponowego może mieć wpływ na funkcję nerwów.

Kolejna badana kwestia to stopień wpływu kwasu alfa-liponowego na funkcję nerwów czuciowych badaną testami przewodnictwa nerwowego.

Dla odpowiedzi na te pytania skonstruowano eksperyment, gdzie postawiono następujące zadania badawcze:

- ocena wpływu dożylnego podawania kwasu alfa-liponowego na funkcję albuminy - stężenia grup SH i karbonyli osocza
- wpływ kwasu alfa-liponowego na przewodnictwo nerwowe w kontekście statusu redoks albuminy.

Dotychczas u pacjentów z neuropatią cukrzycową nie przeprowadzano badań związanych z głównym antyoksydantem osocza – albuminą i jej stanem utlenienia, stężeniami karbonyli i oceną funkcji nerwów.

Ocena statusu redoks albuminy u pacjentów z neuropatią cukrzycową staje się wobec tego wyzwaniem prezentowanej pracy.

MATERIAŁ I METODY

Badanie rozpoczęto po uzyskaniu zgody lokalnej komisji bioetycznej. Pacjenci po wyrażeniu świadomej zgody na udział w badaniu byli rekrutowani w czasie 04.2005-11.2006 z populacji chorych Regionalnego Centrum Diabetologii przy Uniwersyteckim Centrum Klinicznym w Gdańsku. Kryteria włączenia obejmowały: 1. cukrzycę typu 1 lub typu 2, 2. wiek 20-70 lat, 3. obecność bólowej polineuropatii cukrzycowej, 4. leczenie insuliną. Kryteria wyłączenia były następujące: 1. neuropatia niecukrzycowa, 2. palenie tytoniu 3. stosowanie antyoksydantów lub prooksydantów jako suplementów w okresie ostatnich 3 miesięcy 4. choroba naczyń obwodowych 5. istotna choroba układu krążenia 6. schorzenia neurologiczne 8. wysokie średnie glikemie > 400,g/dL lub ketonuria,9. nadużywanie alkoholu, 10. retinopatia proliferacyjna, 11. niewydolność narządowa, 12. dodatkowa ciężka choroba lub niesprawność.

Charakterystykę kliniczną pacjentów włączonych do badania przedstawia tabela:

	Cukrzyca typu 1	**Cukrzyca typu 2**
Liczba pacjentów	13	18
Płeć (M/Ż)	5/8	6/12
Czas trwania objawów polineuropatii – ręce (lata)	4,1±3,0	2,6±2,6
Czas trwania objawów polineuropatii – nogi (lata)	5,8±3,5	5,0±2,9
Czas trwania objawów bólowych – ręce (lata)	2,5±2,4	1,3±2,2
Czas trwania objawów bólowych – nogi (lata)	6,1±3,4	4,4±3,3
Czas trwania leczenia insuliną (lata)	17,9±11,6	8,5±5,1
Dawka insuliny NPH (IU/dobę)	20,0±9,2	22,0±12,3
Dawka insuliny regular (IU/dobę)	29,0±21,2	18,8±18,7
Dawka mieszanki insulin NPH + R (IU/dobę)	40,7±7,0	41,8±14,1
HbA1c (%)	8,6±1,8	7,7±1,1
Wiek (lata)	46,7±7,2	62,8±8,4
Masa ciała (kg)	66,7±11,4	83,2±17,0
Wzrost (cm)	169,2±9,6	167,2±10,0
Wskaźnik masy ciała (BMI)	23,3±3,6	29,7±5,1
Czas trwania cukrzycy (lata)	18,0±11,5	16,5±6,5

Wszyscy pacjenci przeszli 4-tygodniowe szkolenie obejmujące kwestie diety cukrzycowej, metod prowadzenia samokontroli glikemii i samodzielnego dostosowywania dawek insuliny. Indywidualnie optymalizowano leczenie insuliną w oparciu o preparaty ludzkie lub analogowe celem uzyskania zadawalających wyników glikemii na czczo i poposiłkowych. leczenie kwasem alfa-liponowym rozpoczęto po 3-miesięcznym okresie „stabilizacji".

Ocena stanu redoks albuminy polegała na pomiarze stężenia zredukowanej cysteiny 34 za pomocą DTNB oraz stężenia grup karbonylowych.

Wyrównanie metaboliczne pacjentów określano za pomocą stężenia Hba1c.

Funkcja nerwów obwodowych była badana za pomocą badania przewodnictwa nerwowego.

W ramach badań biochemicznych oznaczano stężenia: Hba1c, kreatyniny, potasu, sodu, ASpat, Alat, bilirubiny, Falk, GGTP, składowych lipidogramu, a także oznaczano parametry morfologii, OB. i mikroalbuminurię.

Oznaczenia grup karbonylowych wykonano wg metody Levine, w czasie nieprzekraczającym 2 godziny od pobrania krwi.

Oznaczenia grup SH wykonano wg metody Habeeb.

Badanie neurologiczne obejmowało ocenę objawów neuropatii bólowej takich jak ból, parestezje, drętwienia, a także ocenę odruchów ścięgnistych, siły mięśniowej, czucia bólu i dotyku, czucia głębokiego, czucia wibracji, badanie przewodnictwa nerwowego.

Wszyscy pacjenci wypełniali ankiety – ze skalą bólu VAS, skalą bólu Neuropathy Pain Score, Likert, oraz skalą wpływy bólu na wykonywanie pracy Pharmacoeconomy question.

Procedury badania wykonano wg schematu.

Schemat badania

Tydzień	-12	-1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	5
Wizyta	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		18
			OKRES LECZENIA																
Dzień leczenia			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Świadoma zgoda	x																		
Program edukacyjny	x																		
Okres stabilizacji	x																		
Samokontrola glikemii		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Badanie przedmiotowe	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
Badanie krwi - karbonyle, grupy SH			x (B) dzień 1																x(G) dzień 30
Badanie krwi - biochemia	x(A)		x (B) dzień 1														x(F)		x(G) dzień 30
Badanie krwi - HbA1c			x (0)														x (15)		
Badanie dna oka	x																		
Badanie neurologiczne			x (B) dzień 1																x(G) dzień 30
Kwestionariusze		x (1)															x (2)		x (3)

*Samokontrola glikemii dotyczyła kwestii bezpieczeństwa - monitorowanie celem uniknięcia niedocukrzeń

WYNIKI

W całej badanej populacji stwierdzono obniżenie Hba1c(*p<0,05).

W podgrupie pacjentów z cukrzycą typu 1 spadek nie był istotny statystycznie, najpewniej z powodu umiejętności sprawnego dostosowywania dawek insuliny.

Parametry morfologii krwi, OB., stężenie kreatyniny, Alat, Aspat, bilirubiny, Falk, FGGTP, frakcje lipidów, mikroalbuminuria – nie uległy zmianie.

Ocena parametrów utlenienia albuminy – w całej grupie nie stwierdzono zmian w zakresie stężenia karbonyli i grup SH, natomiast w podgrupie pacjentów z cukrzycą t 1 stwierdzono znamienny ($p = 0.037$) wzrost stężenia grup SH. Podobnie, znamienny ($p = 0.03$) wzrost stężenia grup SH stwierdzono w podgrupie 9 pacjentów pacjentów z cukrzycą 1 i 2 leczonych blokerami kanałów wapniowych (amlodipina, felodipina, werapamil). Nie stwierdzono zależności od innych często stosowanych leków (metforminy, inhibitorów RAA, nitratów, statyn).

Parametry neurologiczne – wykazano istotna poprawę w zakresie szybkości przewodzenia we włóknach ruchowych nerwu pośrodkowego ($p = 0.15$)

W badaniach ankietowych stwierdzono istotne zmniejszenie dolegliwości bólowych oceniane skalą VAS (Visual Analog Scale) (ANOVA test, $p < 0.0001$), oraz NPS i Likert. Poprawa w zakresie pracy zawodowej nie była znamienna.

W czasie leczenia istotnie spadło zapotrzebowanie na leki przeciwbólowe ($p=0.000655$).

Potwierdzono wystąpienie istotnej dodatniej korelacji między stężeniem grup SH a szybkością przewodzenia w nerwie pośrodkowym w całej populacji chorych i osobno u osób z cukrzycą typu 1.

Nie stwierdzono korelacji dla parametrów: : ΔVAS, ΔHbA1c, ΔSH, wzrost, płeć, Δkarbonyl, pozostałe parametry neurologiczne.

DYSKUSJA

Kwas alfa-liponowy jest skutecznym lekiem stosowanym w cukrzycowej polineuropatii bólowej. Jednak jego mechanizm działania pozostaje niejasny. Wykazano, że poziom bólu zależy od statusu redoksowego kanału Cav3.2.

Dotychczas wszystkie mechanizmy bioprotekcyjne kwasu alfa-liponowego przypisywano jego właściwościom antyoksydacyjnym.

Sprzecznie z oczekiwaniami, to jego działanie prooksydacyjne na grupy tiolowe kanałów błonowych powoduje zamykanie kanałów i zmniejszenie odczuwania bólu. Wyniki badań wskazują na zarówno antyoksydacyjne, jak i prooksydacyjne mechanizmy działania kwasu alfa-liponowego.

Farmakologiczne dawki ALA uzyskują stężenia terapeutyczne w nerwach obwodowych dzięki wiązaniu przez cząsteczkę albuminy ludzkiej.

Wydaje się, że kwas alfa-liponowy przyłączając się do domeny albuminy ludzkiej wiążącej kwasy tłuszczowe ma odwrotny niż długołańcuchowe C16 kwasy tłuszczowe wpływ na stan redoks cysteiny 34 albuminy co chroni ja przed utlenieniem i zapewnia dobre warunki dla nitrozylacji cysteiny przez fizjologiczny wazodylatator tlenek azotu NO. (Rola NO jest nanalogiczna jak kanału Cav3.2)

Efektem poprawy stanu redoks Cys34 jest skuteczna transnitrozylacja prowadząca do fizjologicznego rozszerzenia naczyń zaopatrujących nerwy i poprawa funkcji przewodnictwa nerwowego.

Niedawno wykazano, że długołańcuchowe kwasy tłuszczowe mają odwrotne działanie na wiązanie cynku do albuminy. Wskazuje to na możliwość poprawy funkcji albuminy poprzez kwas alfa-liponowy w zakresie przyłączania jonów cynku i dostarczania tego istotnego ligandu do kanału Cav3.2.

Efektem działania jonów cynku jest zamknięcie kanału i zmniejszenie bólu.

Nowe wyniki badań wskazują na to, że indukowane oksydacją zmiany struktury mikrodomen kanału Cav3.2 powodują zamknięcie kanału jonowego, zmniejszając odczuwanie bólu, a utlenienie albuminy powoduje utratę jej funkcji biologicznych. W obu cząsteczkach działanie kwasu alfa-liponowego jest odmienne – utlenia on cysteinę kanału błonowego a redukuje cysteinę albuminy.

W toku leczenia kwasem alfa-liponowym uzyskano poprawę parametrów elektrofizjologicznych znamiennie w zakresie szybkości przewodzenia we włóknach ruchowych i czuciowych nerwu pośrodkowego. Oba statystycznie znamienne wyniki dotyczyły nerwów krótszych, wskazując na tom, że długość uszkodzonego neuronu jest ważnym czynnikiem w patologii cukrzycy i skuteczności leczenia.

W trakcie leczenia kwasem alfa-liponowym w badanej populacji pacjentów z cukrzycą nie stwierdzono różnic w stężeniach karbonyli. Ocena statusu oksydacji białek surowicy przy użyciu karbonyli nie jest dla tej populacji metodą z wyboru. W przeciwieństwie do karbonyli, stężenia grup SH wykazują istotne różnice przed i po leczeniu ALA. Grupy SH mogą być dobrym parametrem oceny statusu redoks u pacjentów z cukrzycą.

Utlenienie grup SH zachodzi wyłącznie w cysteinie 34 – jedynej cysteinie albuminy ludzkiej.

Stężenie grup SH rosło po miesięcznym leczeniu kwasem alfa-liponowym. Poprawa funkcji neuronów zależy od stężenia grup SH cysteiny 34.

Dotychczasowe próby oceny statusu tiolowego albuminy oparte są prawie wyłącznie na badaniu albuminy izolowanej z osocza, z osocza bogatopłytkowego. Metody te są obarczone błędem – izolacja albuminy, jak to wykazał Peter J. Sadler prowadzi do błyskawiczego jej utlenienia i obniżenia stężenia grup SH.

Przy prawidłowym przeprowadzeniu eksperymentu ilość tioli osocza powinna być ekwiwalentna albuminie; natomiast po izolacji stężenie grup SH jest błędnie zaniżone z powodu utlenienia do 50-70%.

OGRANICZENIA PRACY

W trakcie leczenia kwasem alfa-liponowym uzyskano istotne obniżenie wartości Hba1c w całej grupie pacjentów (p = 0,043). W grupie pacjentów z cukrzycą typu 1, obniżenie Hba1c nie było istotne (p = 0,084), natomiast w grupie pacjentów z cukrzycą typu 2 – obniżenie było znamienne statystycznie (p = 0,017). Kwas alfa-liponowy ma wpływ na wrażliwość na insulinę oraz niezależny od insuliny pobór glukozy przez komórki – i to jest jedna przyczyna tych różnic. Z drugiej strony warto podkreślić, ze pacjenci z cukrzycą typu byli przeszkoleni w stosowaniu funkcjonalnej insulinoterapii, dostosowywali dawki insuliny na bieżąco, a biorąc pod uwagę ich większą skłonność do niedocukrzeń mogli redukować dawki insuliny aby osiągnąć optymalne wyrównanie glikemii.

Ala jest naturalnym kofaktorem kompleksu dehydrogenazy mitochondrialnej i silnym antyoksydantem, poprawia on metabolizm glukozy u osób z cukrzycą typu 2 oraz w modelach zwierzęcych cukrzycy. Wykazano, że R (+) kwas alfa-liponowy bezpośrednio aktywuje kinazy tyrozynowe i serynowe/treoninowe, co stymuluje pobieranie glukozy do komórek. Własności te są unikatowe. Opisany jest też mechanizm obniżający glikemię dotyczący receptora GLUT4 w komórce mięśniowej szczurów z cukrzycą indukowaną streptozotocyną.

Hba1c jest znanym klasycznym czynnikiem ryzyka rozwoju neuropatii cukrzycowej.

Stężenie karbonyli po leczeniu kwasem alfa-liponowy nie zmieniło się. Stwierdzono za to wzrost stężenia grup SH, znamienny w grupie pacjentów z cukrzycą typu 1(p = 0.037). Możliwe, że to metabolizm wolnych kwasów tłuszczowych stanowi różnicę między pacjentami z cukrzycą typu 1 i cukrzycą typu 2.

Projekt objął pacjentów poddawanych standardowej terapii. Większość z nich poza insuliną przyjmowała leki takie, jak : gliklazyd (3 z 31), inhibitory enzymu konwertującego(22 z 31), blokery kanału wapniowego i statyny (16 z 31). Część z wymienionych leków zmniejsza stres oksydacyjny, wpływając pośrednio na oznaczenie stężenia grup karbonylowych.

U 9 pacjentów z cukrzycą typu 1 i 2 leczonych blokerami kanału wapniowego (amlodypina, felodypina, werapamil) stwierdzono istotne zwiększenie stężenia grup SH(p = 0.03).

Jak dotychczas nie ma eksperymentalnego dowodu in vivo na aktywność aktyoksydacyjną blokerów kanału wapniowego.

Nie stwierdzono korelacji między parametrami: ΔVAS, ΔHbA1c, ΔSH, wzrost, ΔPMCV, ΔMMCV, ΔMNSV, płeć, Δkarbonyl. Wartość korelacji Pearsona między zmianą szybkości przewodnictwa w nerwie pośrodkowym i przyrostem grup SH wynosiła 0,6 (=0,025).

Histydyna 191 kanału $Ca_v3.2$ ma duże znaczenie w modulacji czynników redoksowych, wiązaniu jonów cynku, i prawdopodobnie także zaopatrywaniu w cynk cząsteczek albuminy.

Określenie statusu cysteiny 34 w toku leczenia kwasem alfa-liponowym daje pośrednią wiedzę na temat zaopatrzenia w cynk kanałów $Ca_v3.2$ neuronów czuciowych lub

obecności kwasu alfa-liponowego związanego z albuminą w łożysku naczyniowym, który usprawnia wiązanie cynku do albuminy.

W cukrzycy przedłużona ekspozycja albuminy na zwiększone stężenia kwasów tłuszczowych w surowicy krwi ogranicza specyficzne dla albuminy wiązanie cynku, co może nasilać objawy neuropatii i depresji.

WNIOSKI

Założone cele pracy zostały osiągnięte.

Uzyskano wyniki badania skutków leczenia kwasem alfa-liponowym względem statusu redoks białek osocza (z których 80%) stanowi albumina u pacjentów z bólową neuropatią cukrzycową. Rezultaty badania pozwalają na głębszy wgląd w elementy patomechanizmu neuropatii.

Potwierdzono korzystny wpływ leczenia kwasem alfa-liponowym na poprawę przewodnictwa nerwowego we włóknach ruchowych i czuciowych nerwu pośrodkowego. Istotna poprawa parametrów przewodzenia dotyczyła krótszych z badanych nerwów, co potwierdza rolę długości nerwu jako istotnego czynnika patogenezy neuropatii i skuteczności jej leczenia.

15-dniowe leczenie dożylne racemicznym kwasem alfa-liponowym w dawce 600 mg/dobę skutkuje istotną poprawą w zakresie zarówno objawów przedmiotowych jak i podmiotowych bólowej neuropatii cukrzycowej.

Poprawa w zakresie objawów wiąże się z poprawą parametrów patofizjologii neuronu.

Istotna poprawa szybkości przewodzenia w nerwach oraz zmiany w zakresie innych parametrów przewodzenia wskazują na silny neurotropowy potencjał leczenia.

W wyniku leczenia przewodnicwto nerwowe może ulega poprawie, co wskazuje na to, że uszkodzenie neuropatyczne nerwów może być chociaż częściowo odwracalne i klinicznie kontrolowane.

Podawanie dożylne kwasu alfa-liponowego wiązało się także z obniżeniem wartości Hba1c, przy czym w grupie pacjentów z cukrzycą typu 2 zależność ta była istotna statystycznie.

W trakcie leczenia nie obserwowano istotnych zmian w stężeniu grup karbonylowych.

Leczenie kwasem alfa-liponowym powoduje zmniejszenie utlenienia albuminy, ale nie chroni jej przed karbonylacją.

Karbonylacja wynika z aktywności redoksowej jonów metali przejściowych, w tym m.in. żelaza. Wyniki wskazują na to, że kwas alfa-liponowy nie jest istotnym chelatorem metali przejściowych w tej grupie pacjentów.

Badania in vitro (nie opublikowane) wykonane na albuminie ludzkiej wykazują, że kwas alfa-liponowy jest znacznie słabszym chelatorem metali przejściowych niż wybrane bioflawonoidy.

Bardzo ciekawym dla dalszych rozważań i badań naukowych wynikiem jest wzrost stężenia grup SH mierzony miesiąc po leczeniu dożylnym kwasem alfa-liponowym, co najpewniej wynika z deoksydacji grup SH cysteiny 34 albuminy ludzkiej. Poprawa funkcji nerwów wiąże się z funkcją grup SH.

Badanie nie dotyczyło statusu albuminy izolowanej, ponieważ proces izolacji powoduje szybkie nieodwracalne utlenienie albuminy. Albumina przechowywana w mrożonym osoczu przez długi czas zachowuje swoje właściwości.

bardzo mała grupa pacjentów leczonych blokerami kanału wapniowego uzyskała znacznie lepsze wyniki w zakresie wzrostu grup tiolowych.

Monitorowanie grup SH może być użytecznym testem diagnostycznym monitorującym leczenie kwasem alfa-liponowym.

Cukrzyca jest chorobą związaną z dużym stresem oksydacyjnym. Zastosowanie określonych antyoksydantów może wiązać się z poprawą rokowania i regresją przewlekłych powikłań cukrzycy.

Leczenie kwasem alfa-liponowym, w odniesieniu do metabolizmu mitochondrialnego i statusu neuronalnego może w latach przyszłych stać się centrum zainteresowania w badaniach nad bólową neuropatią cukrzycową.

Leki spełniające kryterium korzystnego wpływu na szlaki metaboliczne istotne dla rozwoju przewlekłych powikłań cukrzycy są nadzieją na poprawę rokowania u pacjentów z cukrzycą.

PODSUMOWANIE

Dorośli pacjenci z cukrzycą typu 2 byli rekrutowani do projektu od kwietnia 2005 roku do listopada 2006 roku spośród populacji chorych będących pod opieką Regionalnego Centrum Diabetologii Uniwersyteckiego Centrum Klinicznego w Gdańsku.

Badanie odbyło się za zgodą lokalnej Komisji Etycznej.

Uzyskano świadomą zgodę od wszystkich podmiotów uprawnionych do wzięcia udziału w badaniu. Kryteria włączenia obejmowały : cukrzycę typu 1 lub typu 2 (według Światowej Organizacji Zdrowia / American Diabetes Asociation / kryteriów Polskiego Towarzystwa Diabetologicznego) , wiek 20-70 lat, klinicznie ewidentną bólową polineuropatię, leczenie insuliną. Kryteriami wykluczającymi były: 1) neuropatia bez cukrzycy; 2) palenie tytoniu 3) stosowanie przeciwutleniaczy (witaminy C, E, probukol, P-karoten, karwedilol, chelatory żelaza, kwas α-liponowy) lub prooksydanty (żelazo) w ciągu ostatnich 3 miesięcy; 4) objawy podmiotowe choroby tętnic obwodowych ; 5) objawową chorobę niedokrwienną serca, stan po zawale mięśnia sercowego, niewydolność serca, stan po wszczepieniu rozrusznika serca; 6) przyjmowanie leków wpływających na funkcje neurologiczne; 7) choroby neurologiczne; 8) stężenie glukozy we krwi> 400 mg / dl i / lub ketonurię; 9) nadużywanie alkoholu; 10) obecność retinopatii proliferacyjnej; 11) wszelkie niewydolności systemowe; 12) każdą poważną chorobę.

Wpływ leczenia za pomocą kwasu alfa-liponowego badano prospektywnie u 31 pacjentów z cukrzycą. Wszyscy pacjenci otrzymywali kwas alfa-liponowy (Thiogamma, Wörwag) dożylnie w dawce 600 mg na dobę przez 15 dni.

Badano parametry stresu oksydacyjnego - stężenie grup SH i stężenie karbonyli w surowicy. W tym samym czasie przeprowadzono badania przewodnictwa nerwowego i ocenę czucia bólu.

Prędkość przewodzenia we włóknach czuciowych i ruchowych, progi czucia wibracji, HbA1c, stężenie tioli i grup karbonylowych w osoczu mierzono na początku badania, po 15 dniach leczenia oraz 15 dni po zakończeniu leczenia.

W wyniku leczenia uzyskano poprawę w zakresie wielu parametrów przewodnictwa nerwowego, przy czym poprawa szybkości przewodzenia we włóknach ruchowych nerwu pośrodkowego była istotna.

Zaobserwowano spadek HbA1c w całej badanej populacji chorych na cukrzycę typu 1 i cukrzycy typu 2 z bolesną neuropatią.

W grupie z cukrzycą typu 1 (n = 13), zmniejszenie HbA1c nie było znaczące (p = 0,067). (T0 – przed leczeniem, T1- po 15 dniach leczenia dożylnym kwasem alfa-liponowym w dawce 600 mg). Brak istotności wynika najpewniej z dostosowywania dawek insuliny celem uniknięcia niedocukrzeń, łatwiejsze u dobrze wyedukowanych pacjentów z cukrzycą typu 1.

W grupie z cukrzycą typu 2 (n = 17), zmniejszenie HbA1c było istotna (p = 0,0074). (T0 – przed leczeniem, T1- po 15 dniach leczenia dożylnym kwasem alfa-liponowym w dawce 600 mg). Efekt był znaczący prawdopodobnie ze względu na stabilne dawkowanie insuliny.

Zmianę stężenia grup SH białek osocza zaobserwowano zwłaszcza u pacjentów z cukrzycą typu 1 - była znacznie wyższa w porównaniu do stanu wyjściowego (* p <0,05).

W toku leczenia kwasem a-liponowym nie zaobserwowano zmian w stężeniach białek osocza.

Objawy bólu, opisywane przez pacjentów za pomocą skali VAS (Visual Analog Scale) (test ANOVA, p <0,0001) (* p <0,05 w porównaniu do B) i skali Likerta (* p <0,05) uległy znacznemu zmniejszeniu w trakcie leczenia kwasem alfa-liponowym.

Wyniki wskazują na selektywny korzystny wpływ kwasu alfa-liponowego na stan utlenienia albuminy surowicy. Ze względu na fakt, że cysteina34 albuminy ludzkiej jest głównym nośnikiem tlenku azotu to przypuszcza się, że ochrona tioli cysteiny może być ważną częścią neuroprotekcyjnego działania kwasu liponowego.