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The Role of Oxidative Stress in Pathogenesis of Diabetic Neuropathy: Erythrocyte Superoxide Dismutase, Catalase and Glutathione Peroxidase Level in Relation to Peripheral Nerve Conduction in Diabetic Neuropathy Patients

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1. Introduction

Distal symmetrical polyneuropathy is the most common form of neurological complications in diabetes mellitus (DM). It is sensory-motor polyneuropathy dominated by sensitive symptoms and signs, primarily decrease or loss of sensibility in distal parts of extremities, or positive symptoms of prickling, burning pain and tingling. These symptoms can be extremely unpleasant for patients but at the same time they are a huge therapeutic problem as well. Motor symptoms and signs in early phase of DSP are often absent, although by neurophysiologic tests motor fibers damaging signs can be detected and this damage often leads to loss of functions in patients in advanced phase of the disease. The most significant clinical consequence of sensitive and autonomic fibers damage is feet ulceration, what is a leading cause for hospitalization and for lower extremities amputations, if trauma factors are excluded. Thus, diabetic DSP is important not only for clinical reasons but for economic ones as well, especially regarding the fact that DM prevalence is rapidly growing. For many years scientists have done numerous researches for therapeutic solutions to prevent, delay or slow the progression of the disease, aiming to better understanding of ethiopathogenesis of this disease. Special attention has been paid to oxidative stress (OS) role in pathogenesis of DM and diabetic neuropathy. Despite numerous experimental confirmations of OS in diabetic patients, there is still controversy about whether oxidative stress is just a side effect or it is a possible cause of diabetic neuropathy. More than thousand studies about this problem have already been reported and since this problem is not completely solved, further investigations are required. Therefore, a good knowledge of biochemistry of oxidative stress, cell signal transduction and antioxidative protection is necessary.

2. Biochemistry of oxidative stress

Oxidative stress is a consequence of the imbalance between reactive oxygen species (ROS) production and antioxidant capacity. This can occur as a result of either increased ROS
generation, impaired antioxidant system, or a combination of both. Free radicals are defined as atoms or molecules that contain one or more unpaired electrons, making them unstable and highly reactive (Halliwell, 1999). The most important free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS include free radicals such as superoxide (O$_2^-$), hydroxyl (OH), peroxyl (RO$_2^-$), hydroperoxyl (HRO$_2^-$) as well as nonradical species such as hydrogen peroxide (H$_2$O$_2$) and hydrochlorous acid (HOCI) (Turko et al., 2001; Evans et al., 2002; Ziegler, 1994; Soliman & Gellido, 2004). Other non-oxygen species existing as reactive nitrogen species (RNS) include free radicals like nitric oxide (NO) and nitrogen dioxide (NO$_2$), as well as nonradicals such as peroxynitrite (ONOO$^-$), nitrous oxide (HNO$_2$) and alkyl peroxynitrates (RONOO$_2^-$) (Ziegler, 1994; Soliman and Gellido, 2004).

In aerobic cells, free radicals are constantly produced mostly as reactive oxygen species. Once produced, free radicals are removed by antioxidants defenses including enzyme superoxide dismutase, catalase and glutathion peroxidise. Reactive oxygen species, including nitric oxide and related species, commonly exert a series of useful physiological effects. However, imbalance between prooxidant and antioxidant defenses in favour of prooxidants results in oxidative stress associated with the oxidative modification of biomolecules such as lipids, proteins and nucleic acids.

Hyperglycemia is the main cause of increased concentration of free radicals in the plasma of diabetic patients. Chronic hyperglycemia causes oxidative stress (OS) in a number of ways including enzymatic, non-enzymatic and mitochondrial pathways, thus disrupting the prooxidative/antioxidative balance in cellular systems. Nonenzymatic sources of oxidative stress originate from the oxidative biochemistry of glucose. Hyperglycemia can directly cause increased ROS generation. Glucose can undergo autoxidation and generate OH radicals. A second consequence of hyperglycemia is nonenzymatic glycation of proteins.

Nonenzymatic glycation of proteins implies the ability of glucose to react in a nonenzymatic process with proteins, reducing molecular oxygen to highly reactive products (superoxide radical, hydrogen peroxide, and hydroxyl radical). The glycation process occurs in two phases: early – reversible, and late – irreversible phase. The early phase is characterized by the development of Amadori products followed by formation of stable adducts - advanced glycation end products (AGEs) in the later phase. The production of intracellular AGE precursors damages target cells via three pathways. First, intracellular proteins, modified by AGE, alter their own function. Second, extracellular matrix components modified by AGEP precursors interact abnormally with other matrix components and with matrix protein receptors on the cells. Third, plasma proteins modified by AGE precursors bind to the AGE receptors on endothelial cells, mesangial cells, and macrophages, causing receptor-mediated production of reactive oxygen species (ROS).

Enzymatic sources of augmented generation of reactive species in diabetes include NOS, NAD(P)H oxidase and xanthine oxidase (Guzik et al., 2000; Guzik et al., 2002; Aliciguzel et al., 2003), where it is considered that the NAD(P)H oxidase is the major source of O$_2^-$ production (Guzik, 2000; Ergul et al., 2004).

The mitochondrial respiratory chain is another source of nonenzymatic generation of reactive species. Hyperglycemia-induced generation of O$_2^-$ at the mitochondrial level is the initial trigger of vicious cycle of oxidative stress in diabetes (Schultz et al., 2005, Nishikawa et al., 2000, Brownlee, 2001). Increased generation of ROS, especially O$_2^-$ precedes the activation of four major pathways involved in the development of diabetic complications.
In hyperglycemia there is enhanced metabolism of glucose through the polyol (sorbitol) pathway, which also results in enhanced production of \( \text{O}_2^- \). Glucose is a poor substrate for aldose reductase, but at high concentrations this enzyme converts glucose to sorbitol, initiating the polyol pathway of glucose conversion to fructose. This pathway of glucose metabolism implies the participation of aldose reductase enzyme, which using the NADPH cofactor, catalyzes the reduction of glucose into sorbitol, which can be further transformed into fructose. Aldose reductase is widespread in the mammalian tissues such as peripheral nerves, retina, renal glomeruli, eye lenses. Stimulation of this pathway in hyperglycemic conditions is one of the pathogenetic mechanisms responsible for the development of diabetic neuropathy, nephropathy, and cataract. Activation of this pathway leads to structural changes in the tissues via several mechanisms, out of which most significant are increased osmotic pressure in the cell, depletion of myoinositol, and disordered redox potential of the cell due to reduced NADPH concentration, on the account of which the activity of NADPH-dependent enzymes (including glutathione reductase and NO synthase) is diminished. Reduced production of NO leads to vasoconstriction, and impossibility of reduced glutathione regeneration in the so-called glutathione redox cycle leads to depletion of reduced glutathione, since it is regenerated by the action of NADPH-dependent glutathione reductase (Garlberg & Mannervik, 1975), ultimately producing a permanent oxidative stress. Change of redox status of the cell, as determined by the cellular content of thiol compounds, glutathione above all, is a significant factor in the regulation of signal transduction to appropriate genes. This signal transduction pathway is effectuated via so-called redox sensors which react to any change of concentration of intracellular thiols responsible for upkeep of the cellular redox status. Numerous regulatory transcription factors suppress transcription if bound in reduced state to regulatory sequences of certain genes. In the absence of GSH in the cell and in the state of intensified polyol metabolic pathway, the oxidized form of regulatory nuclear transcription factors loses the affinity for regulatory gene sequence, which results in increased transcription and synthesis of appropriate functional and structural proteins responsible for complications in diabetes and dysregulation of glucose metabolism homeostasis. Disturbed redox potential initiates the activation of stress-signaling cascade, resulting in direct activation of other kinases and transcription factors and/or indirect modulation (oxidation) of cysteine-rich redox-sensitive proteins, such as thioredoxin and glutathione S-transferase (Adler et al., 1999).

2.1 Modulation of cell signal transduction

Results of recent clinical and experimental in vivo and in vitro studies unequivocally suggest that DM is a disease followed by intensified oxidative stress which modulates numerous cell transduction pathways (Pavlović et al., 2002; Tomlinson & Gardiner, 2008). This eventually results in tissue damage and the emergence of numerous diabetic complications, including peripheral neuropathy. Hyperglycemia leading to oxidative stress induces diabetic complications via two signaling pathways: activation of protein kinase C (PKC) (Tomkin, 2001), and activation of mitogen-activated protein kinases (MAPK) (Tomlinson, 1999). PKC represents a family of multifunctional enzymes synthesized on at least three separate genes. They play a fundamental role in signal transduction in various tissues via phosphorylation (up and down regulation) of enzymes, receptors, transcription factors, and other kinases. PKC family consists of at least 11 isoforms, out of which 9 are activated by way of lipid secondary
messenger diacylglycerol (DAG). High glucose concentration increases the level of DAG stimulating de novo synthesis of DAG, which activates PKC. Hyperglycemia can also activate PKC isoforms via AGE receptors (Portilla et al., 2000) and by increased activity of the polyol pathway (Keogh, Dunlop & Larkins, 1997), probably by increasing reactive oxygen species (ROS). Intensified DAG-PKC signaling pathway is responsible for the disorders which represent the basic mechanisms in the development of vascular complications in diabetes mellitus.

In the complex process of signal transduction there are interactions of numerous receptor proteins and impulses are being amplified, so that joint, cascade pathways of signal transduction may be talked about. One of these pathways is the PKC-MAPK signal transduction pathway (Tomlinson & Gardiner, 2008). Specific isoforms of PKC can activate MAPK, which via phosphorylation of transcription factors lead to altered expression of certain genes, making conditions for the change of cellular phenotype, apoptosis, or survival with a resultant disbalance producing complications in particular organs and systems. Thus MAPK activation represents a key event in the development of diabetic complications, especially diabetic neuropathy. Activation of this signaling pathway induces changes at the level of ion channels or disturbed gene expression, directly leading to nerve conduction disorders and development of axonopathy.

MAP kinases are group of serin/threonine specific kinases which are activated in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. There are three main groups of MAP kinases: extracellular signal-regulated protein kinases (ERK), p38 and c-Jun N-terminal kinases (JNK). Results of recent clinical and experimental in vivo and in vitro studies show that all three groups of MAP kinases are activated in sensory neurons in hyperglycemia conditions in both diabetic rats and patients. (Purves et al., 2001). In general the JNK and p38 group mediate responses to osmotic stress and is likely to be involved in the regulation of aldose reductase expression. Activation of p38 MAPK results in generating reduced nerve conduction velocity (NCV). These changes in NCV results from phosphorylation of the sodium channel Na1.6 (Wittmack et al., 2005), which is the main voltage-activated channel at the node of Ranvier in myelinated fibres. In later stages of DM, other factors prevail: neurofilaments perturbation occurs, as a result of JNK activation and hyperphosphorylation of neurofilament proteins.

2.2 Antioxidant protection

The biological oxidative effects of free radicals on macromolecules are controlled by a spectrum of enzymatic and nonenzymatic antioxidants. Intracellular antioxidative defense is primarily enabled by the antioxidative enzymes, most significant of which are SOD, CAT and GSH-Px (Andrea et al., 2004; Maritim et al., 2003; Szaleczky et al., 1999; Djordjević, 2004). These three antioxidant enzymes differ in their structure, tissue distribution and cofactors required for functioning, SOD works as the first line of enzymatic protection against superoxide radicals. This enzyme catalyses the dismutation of superoxide anion radicals into hydrogen peroxide and oxygen. Hydrogen peroxide is further metabolized by CAT and GSH-Px, and due to lower Km values, GSH-Px is active in lower concentrations of hydrogen peroxide, while CAT activity increases with increased hydrogen peroxide concentration (Djordjević, 2004; Djordjević et al., 2000). Glutathione, together with its related enzymes, comprises a system that maintains the intracellular reducing environment and acts as primary defense against excessive generation of harmful ROS.
During reduced antioxidative protection and/or increased production of free radicals, OS occurs, playing an important role as a mediator of the apoptosis of both neurons and supportive glial cells, which has been confirmed in studies using animal models and tissue cultures (Schmeichel et al., 2003; Russel et al., 1999; Russel et al., 2002; Polydefkis et al., 2003; Person et al., 2003; Chiarelli et al., 2000; Sakaue et al., 2003).

2.2.1 Superoxide dismutase (SOD)

SOD catalyzes the conversion of superoxide anion into hydrogen peroxide and oxygen.

\[
O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

SOD activity was originally described by McCord and Fridovich in 1969 (Szaleczky et al., 1999); they subsequently established that this enzyme is essential in sustaining life in aerobic conditions (McCord et al., 1971). There are several forms of SOD. These are metalloproteins, each containing a metal ion in its center (CuZn SOD, Mn SOD, Fe SOD, and Ni SOD) (Fridovich, 1998). Fe SOD can be found in prokaryotes and plants, while in humans intracellular CuZn SOD as well as mitochondrial Mn SOD were identified. Intracellular CuZn is composed of two identical subunits (Tainer et al., 1982), each containing Cu (II) and Zn (II). Cu (II) is responsible for catalytic activity of SOD, while Zn (II) has a role in the stabilization of enzyme conformation (Djordjević, 2004). Cytosolic CuZn SOD can be inactivated by hydrogen peroxide, leading to the formation of either Cu (II) – OH or the ionized form Cu (II) –O. This enzyme can further catalyze peroxidation of various substances. The gene coding for CuZn SOD is located on chromosome 21. In connection with that, the results of some studies have demonstrated a significantly elevated activity of this enzyme in the patients with Down syndrome (Kurobe et al., 1990).

Extracellular SOD (ECSOD) is structurally similar to intracellular SOD, but it is present in the extracellular space. The gene for ECSOD is located on the chromosome 4. ECSOD is a tetrameric glycoprotein, each subunit of which contains a Cu and Zn atom and has a high affinity for heparin sulphate, enabling its existence in relatively high concentrations in specific regions of extracellular space or on the cell surface. ECSOD expression is principally regulated by cytokines, such as IFNγ, which stimulates the enzyme expression, while TNFα and TGFβ reduce its expression (Marklund, 1992). Reduced ECSOD expression lead to reduced mitochondrial GSH and increased oxidative stress (Lebovitz RM et al., 1996). ECSOD is also significant in modulating NO activity. The superoxide can react with NO and form peroxynitrite, which by way of dissociation can form both hydroxyl radical and nitric dioxide, potent oxidants (Fukai T et al., 2000; Oury TD et al., 1996).

Mitochondrial SOD (MnSOD) is located in the mitochondrial matrix. There are two isoforms of MnSOD, dimeric MnSOD and tetrameric MnSOD, each subunit of which contains one Mn (III) ion. It is produced in a constitutive manner, but can also be induced by IL-1, TNF, or an endotoxin (Tang et al., 1994). In addition to cytokines, numerous oxygen metabolites can also induce MnSOD expression in particular cell types, which can be of critical importance in the occurrence of tissue damage in the situation of oxidative stress (Djordjević, 2004; Yoshioka T et al., 1994). It is believed that transcriptional regulation of MnSOD is mediated by the activation of nuclear transcription factor κB (NF-κB), supported by oxidants. Congenital complete inactivity of MnSOD lead to lethal outcome within few days after birth due to renal dysfunction (Lebovitz RM et al., 1996).
2.2.2 Catalase (CAT)
Catalase is a homotetrameric enzyme found in the tissues of almost all mammals, and demonstrating the highest activity in the liver and erythrocytes. Within the cell, catalase is localized primarily in the peroxisomes and mitochondria. The principal role of catalase lies in the degradation of $H_2O_2$ produced with support of peroxisomal oxidases. In the erythrocytes, catalase constitutes the first line of defense against $H_2O_2$ (Mueller et al., 1997).

In the situations of normal $H_2O_2$ concentration, the reaction goes towards $H_2O_2$ conversion into water and oxygen:

$$\text{Catalase} + 2 \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O}$$

$$\text{Compound I} + \text{H}_2\text{O}_2 \rightarrow \text{Katalaza} + \text{H}_2\text{O} + \text{O}_2$$

The second $H_2O_2$ molecule serves as the donor of hydrogen ion. In the situations of low $H_2O_2$ concentration and in the presence of small molecular electron donors, catalase may act as peroxidase as well (Ghadermarzi & Moosavi-Movahedi, 1996). Catalase forms a firm bond with NADPH, which can prevent the accumulation of inactive forms of the enzyme slowly created when catalase is exposed to hydrogen peroxide. These effects of NADPH are evident in low concentrations in the cell as well ($<0.1$ μM) (Kirkman et al., 1987).

Numerous diseases can be accompanied with altered catalase activity. Reduced catalase activity has been documented in diabetes mellitus (DM), malignant diseases, Down syndrome, as well as in regenerating tissues and in experimental conditions of nephrotoxicity (Djordjević et al., 2000). Since catalase has a predominant role in the control of $H_2O_2$ concentration (Mueller et al., 1997; Gaetani et al., 1996), which has been shown to damage pancreatic β cells and inhibits insulin activity (Murata et al., 1998; Tiedge et al., 1998; Jorns et al., 1999), it is believed that catalase in that manner protects β cells from the harmful action of $H_2O_2$ (Murata et al., 1998).

In the literature, two categories of genetic deficiency of erythrocyte catalase have been described (Eaton and Ma, 1995): acatalasemia (<10% of normal activity) and hypocatalasemia (about 50% of normal activity). In Hungary, a family with acatalasemia and 12 families with hypocatalasemia have been reported (Góth & Eaton, 2000; Góth, 1992; Vitai & Góth, 1997). These families comprised 2 acatalasic individuals, 61 individuals had hypocatalasemia, while 66 individuals had normal values of catalase. In the group with hereditary catalase deficiency, the incidence of diabetes was 12.7%. DM was diagnosed in 8 persons, in both acatalasic and 6 persons with hypocatalasemia; all of them had DM type 2. None of those with normal catalase values had DM. These data can indicate the significance of catalase in the pathogenesis of DM.

2.2.3 Glutathione peroxidase (GSH-Px)
GSH-Px is a selenium-dependent enzyme naturally occurring in three isoforms. The so-called “classical” GSH-Px is a homotetramer with a molecular mass of 80 kDa, containing four atoms of selenium. GSH-Px demonstrates a special affinity for hydrogen peroxide and glutathione (Djordjević, 2004). It reduces $H_2O_2$ and organic alkaline hydroperoxides into water and appropriate alcohols, and oxidizes GSH into an appropriate disulfide (GSSG).

$$2\text{GSH} + \text{H}_2\text{O}_2 (\text{ROOH}) \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O}$$
GSSG is converted back into GSH by glutathione reductase, which utilizes NADPH for its activity. It is thought that NAD(P)H, by itself, as an antioxidant, can act as a scavenger of toxic free radicals, as well as the radicals originating from repaired biomolecules (Kirsch and De Groot, 2001). The classical, selenium-dependent GSH-Pxs, isolated from various organs (erythrocytes, liver, lungs), demonstrate similar features and catalytic properties regardless of their origin. The maximum rate of reaction of GSH-Px depends on the concentrations of GSH and \( \text{H}_2\text{O}_2 \). In contrast to catalase, GSH-Px demonstrates a higher affinity for the substrate (\( \text{H}_2\text{O}_2 \)) at low \( \text{H}_2\text{O}_2 \) concentrations, since the Michaelis constant (\( K_M \)) of peroxidase is lower than that of catalase. That is why GSH-Px plays a key role in the tissue detoxication from \( \text{H}_2\text{O}_2 \) at low \( \text{H}_2\text{O}_2 \) concentrations and normal GSH concentrations, while catalase is actively involved at higher \( \text{H}_2\text{O}_2 \) concentrations in the cell. The role of GSH-Px in erythrocytes is predominantly associated with detoxication from organic hydroperoxides and with keeping in reduced state the SH groups of structural and functional erythrocyte proteins, while its role in \( \text{H}_2\text{O}_2 \) degradation is of a lesser significance compared to catalase. The activity of erythrocyte selenium-dependent GSH-Px correlates with the erythrocyte content of selenium, so that the activity of this enzyme can be used as a functional index to assess selenium deficiency.

The second type of peroxidase is extracellular GSH-Px, with a molecular mass of 21.5 kDa. It is a tetramer too, the subunits of which contain an atom of selenium each. It is thought that extracellular GSH-Px is most important regarding the reduction of \( \text{H}_2\text{O}_2 \) in the plasma. GSH-Px in the plasma is a glycoprotein, believed to be most probably a secretory enzyme released from organ cells into the plasma. This enzyme demonstrates significant kinetic differences compared to erythrocyte GSH-Px. In contrast to erythrocyte GSH-Px, GSH-Px in the plasma demonstrates saturation kinetics compared to GSH (Đorđević et al, 2000). The activity of this enzyme declines with increased glutathione via the value of saturation concentration for GSH of 5 mmol. Moreover, GSH-Px in the plasma demonstrates higher affinity for organic hydroperoxides and \( \text{H}_2\text{O}_2 \). It is thought that selenium-dependent plasma GSH-Px is responsible for the process of detoxication from \( \text{H}_2\text{O}_2 \) and organic hydroperoxides created in the process of synthesis of eicosanoids in entothelial cells (Đorđević et al, 2000). Although the concentration of reduced glutathione in the plasma is very low, it is conceivable that the change in glutathione concentration (interorgan distribution, release from the liver into the plasma) can modulate the enzyme activity. The third type of peroxidase is phospholipid hydroperoxide GSH-Px (PHGpx). It is too a selenium-dependent enzyme which differs from the first two types in view of the substrate and localization. PHGpx is a monomer, with the molecular mass of 23 kDa. This enzyme has a special characteristic – it directly reduces lipid hydroperoxides in the membranes.

Reduced GSH-Px activity can be found in numerous pathologic conditions and diseases, such as in chronic etilism, chronic renal insufficiency, hypertension, endemic nephropathy (Đorđević et al 1998). Altered GSH-Px activity can also be detected in DM (Kaji et al., 1985; Cser et al., 1993; Matkovics et al., 1982).

GSH-Px activity in the nervous system is low (in both peripheral nerves and in the central nervous system). Low values of GSH-Px compared to catalase activity in the peripheral nerves could lead to the conclusion that this enzyme has a minor role in \( \text{H}_2\text{O}_2 \) inactivation. However, GSH-Px function is prominent in the mitochondria, where the enzyme is necessary for the inactivation of \( \text{H}_2\text{O}_2 \) generated during the electron transport (McClain and Crook, 1996) where the catalase activity is insufficient.
2.2.4 Glutathione (GSH)

Glutathione is one of the most important nonenzymatic antioxidant in mammal cells. This ubiquitous tripeptide (γ-Glu-Cys-Gly) accounts for 90% of total non-protein sulphhydril compounds in the cell. In the cell, it is most commonly found as a thiol – in a reduced form – and less as a disulfide – in an oxidized form (Toyokuni, 1996; Hann et al., 1990; Natarajan, 1995).

GSH concentration in the cell is determined by the control of enzymes involved in its synthesis, availability of synthesis precursors, intensity of GSH depletion for cellular detoxification processes, interorgan GSH distribution, as well as GSH regeneration in the so called glutathione redox cycle.

Erythrocytes represent a unique transport system for glutathione and its conjugates. In contrast to other cells, GSH appears in erythrocytes in several intermediary metabolic forms. In physiologic conditions, reduced GSH form appears in the highest percentage. The other GSH form is oxidized glutathione (GSSG), occurring in the process of nonenzymatic oxidation or oxidation mediated by GSH-peroxidase. The third erythrocyte form is the disulphide form of glutathione bound to proteins and non-protein sulphhydril compounds. Glutathione S-conjugates (occurring via the action of glutathione S-transferase) are the fourth potential intracellular-intermediary form of GSH in the erythrocytes. Erythrocytes take up toxic molecules from the plasma, to be excreted back into plasma after conjugation with GSH. Further detoxification of S-conjugates continues in the liver and kidneys; nontoxic compounds are then excreted from the organism via the bile or urine.

GSH is involved in many cellular functions. Many cells synthesize GSH de novo by γ-glutamyl transferase, forming firstly a γ-peptide bond between one cystein and one glutamate residue. The next phase is the addition of glycine, assisted by GSH synthetase. Neurons do not contain γ-glutamyl-cystein synthetase; instead, GSH is synthesized in the glial cells controlling this synthesis via the mechanisms of transcription regulation (Iwata-Ichikawa et al., 1999; Keelan et al., 2001). It is thought that during the evolution, depending on the cellular metabolic needs, glutathione is engaged in the regulation of different processes. Though the role of glutathion has been commonly associated with the protection of the cell from active free radicals, glutathione is involved in many other processes such as detoxification from xenobiotics, synthesis of eicosanoids, synthesis of nucleic acids and proteins, cell signaling, proliferation, and differentiation (Djordjević, 2000). However, its essential role lies in the protective antioxidant system. GSH, GSH-Px, GSH-transferase, GSH-reductase, and NAD(P)H constitute an antioxidant system of glutathione, in which GSH-reductase and NAD(P)H are required for the reduction of oxidized glutathione and consequential glutathione recycling in the so called glutathione redox cycle. Depletion of GSH in the cell increases its sensitivity to oxidative damage (Rizzardini et al., 2003). In contrast, accumulation of GSH in the cell, especially in the mitochondria, can prevent neural apoptosis caused by ischemia (Li et al., 2002) and excitotoxicity (Kobayashi et al., 2000). The involvement of GSH in the limitation of prooxidative cell status, has as the ultimate consequence deceleration of aging, atherogenesis, mutagenesis, and cancerogenesis (Anghileri and Thouvenot, 1997; Vostreis et al., 1988)

3. Aim of study

Research in patients with diabetic neuropathy (DN) is mainly based on studying the influence of antioxidative substances on certain biomarkers of oxidative stress and on the
function of peripheral nerves (Ziegler et al., 1995; Reljanović et al., 1999; Ziegler et al., 1999; Ametov et al., 2003; Ziegler and Gries, 1997; Ziegler et al., 1997). However, there are no enough studies, testing the direct correlation between prooxidative/antioxidative parameters and the development of DN, especially in humans. Additional difficulties occur when different kinds of reactive oxygen are directly determined in biological systems due to their short life. For this reason, the measurement of oxidative stress is mainly based on indirect and non-specific measurement of products of their activity. Considering that antioxidant enzymes are important biomarkers of oxidative stress, the aim of this study was to determine the activities of antioxidant enzymes (SOD, CAT, GSH-Px) and glutathione in the erythrocytes of patients with type 2 diabetes mellitus (DM) in relation to presence or absence of distal symmetrical polyneuropathy (DSP), as well as to analyze the possible connection between the activity of these antioxidant parameters and the function of peripheral nerves. In this way we tried to determine the level of antioxidant defense in erythrocytes of diabetic patients and to establish the potential role of oxidative stress in the development of diabetic neuropathy.

4. Materials and methods

The research took the form of a prospective study, which included 100 patients suffering from type 2 DM and diabetic distal symmetric polyneuropathy (DDSP). Patients suffering from another acute or chronic illness, patients previously subjected to cytotoxic therapy or radiotherapy, and patients who had been treated with antioxidative substances were excluded from the experimental group. The control group gathered 50 healthy individuals who denied ailments and diseases and whose clinical observations and laboratory tests showed no abnormalities. The DDSP was diagnosed after clinical and electrophysiological testing.

The electrophysiological testing checked the conductivity of sensory and motor fibers of upper and lower extremity peripheral nerves. Due to the symmetric nature of the disease, the protocol included the unilateral (right) testing of sural, peroneal, tibial, ulnar and median nerves. We analyzed the latency, amplitude, and conduction velocity of the tested nerves. The minimal criterion for the electrophysiological validation of diabetic neuropathy (DN) was the abnormality of any electroneurographic (ENG) conduction parameter in at least two nerves, one of which had to be sural nerve (England et al., 2005). The values of tested electroneurographic parameters were expressed as the score from 1 to 4, where 1 corresponded to a normal result, while 4 meant that the motor or sensory evoked potential was absent. The ENG testing was carried out on the ENG device (Schwartzer, Mios 2+). During the testing, the surface electrodes were used: the stimulation electrode – for the electrical stimulation of the peripheral nerve and the registration electrode – for the registration of the motor evoked potential (MEP). In order to increase the diagnostic sensitivity of the electrophysiological tests, in addition to the standard ENG, we also tested the conduction sensitivity of the median planter nerve. Since the sensory evoked potential (SEP) amplitude of medial plantar nerve is distinctly low, a separate technique was used to test this on an evoked potential device (Medelec Sapphire 2). A large number (236) of stimuli were used and the values were subsequently averaged by the computer. The following parameters were analyzed: the peak latency of the sensory neurogram, expressed
in milliseconds, and the amplitude of the sensory neurogram, expressed in µV. The stimulation was carried out with a surface electrode positioned on the plantar part of the thumb. The response was registered via a surface electrode located behind the internal malleolus, proximal to the retinaculum flexorum.

Laboratory analysis determined the level of morning glycemia and glycosylated hemoglobin (HbA1c) by the standard laboratory tests from the venous blood of patients and healthy persons. Erythrocyte SOD activity was determined by a commercial test Ransod provided by Randox (Randox Laboratories, Crumlin, UK), based on the McCord and Fridovich method (Mccord and Fridovich, 1969). Erythrocyte CAT activity was determined by the method of Beutler (Beutler, 1982). Erythrocyte GSH-Px activity was determined by commercial test Ransel (Randox Laboratories, Crumlin, UK). Glutathione was measured as total glutathione in the erythrocytes as described by Tietze (1969). Caiman’s GSH Assay kit utilizes a carefully optimized enzymatic recycling method, using glutathione reductase for quantification of GSH.

Statistical method: To process results we used widely accepted statistical techniques: means and standard deviation, statistical significance calculation tests, correlation tests. We utilized standard statistics software tools (Origin Pro and MATLAB Statistics Toolbox). The results are presented as mean ± SD.

5. Results

The study encompassed 100 type 2 DM patients who showed signs of distal symmetrical polyneuropathy, whose average age was 58.62±11.62 years. The average duration of the disease was 11.32±7.05 years. The control group included 50 healthy individuals, whose average age was 51.64±12.25 years (Tab. 1). There was significant increase in glycemia and HbA1c values in the patients compared with the control group (*p<0.0001).

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Sex(M/F)</th>
<th>Age (years) mean ±SD</th>
<th>Duration of DM (years)</th>
<th>Glycemia (mmol/L) mean ±SD</th>
<th>HbA1C (%) mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Patients without DSP</td>
<td>50</td>
<td>22 / 28</td>
<td>51.64±12.25</td>
<td>0</td>
<td>4.81 ±0.63</td>
<td>5.73 ± 0.56</td>
</tr>
<tr>
<td>Patients with DSP</td>
<td>40</td>
<td>24 / 16</td>
<td>57.73±11.08</td>
<td>11.23±7.94</td>
<td>8.99±3.11*</td>
<td>9.26 ± 3.33*</td>
</tr>
<tr>
<td>Patients with DSP</td>
<td>100</td>
<td>55 / 45</td>
<td>58.62±11.62</td>
<td>11.32±7.05</td>
<td>9.50 ±4.13*</td>
<td>9.09 ±2.13*</td>
</tr>
</tbody>
</table>

Table 1. Demographic and biochemical characteristics of diabetic neuropathy patients and the control group.

There was a statistically significant decrease SOD and GSH-Px activity and GSH level in erythrocyte of diabetic neuropathy patients compared with the control group and patients without DN. The values of erythrocyte CAT were lower in patients compared to the control ones, but this difference did not reach statistical significance (Tab. 2).
The Role of Oxidative Stress in Pathogenesis of Diabetic Neuropathy: Erythrocyte Superoxide Dismutase, Catalase and Glutathione Peroxidase Level in Relation to...

<table>
<thead>
<tr>
<th></th>
<th>SOD (U/gr Hb) mean ±SD</th>
<th>CAT (U/gr Hb) mean ±SD</th>
<th>GSH-Px(U/gr Hb) mean ±SD</th>
<th>GSH (µmol/grHb) mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1238.36 ± 136.86</td>
<td>7.34 ± 1.62</td>
<td>67.35 ± 11.39</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>Patients without DSP</td>
<td>1144.26 ± 103.92**</td>
<td>7.22 ± 2.01</td>
<td>55.63 ±13.07**</td>
<td>0.22 ± 0.05**</td>
</tr>
<tr>
<td>Patients with DSP</td>
<td>1101.00 ± 64.36**,*</td>
<td>6.68 ± 1.26</td>
<td>50.25 ± 10.42**</td>
<td>0.21 ± 0.04**</td>
</tr>
</tbody>
</table>

Table 2. SOD and CAT in the diabetic neuropathy patients and in the control group. ** p<0.001 vs. control, * p<0.05 vs. patients without DN

Values of antioxidant parameters were variable at different stages of the disease in patients with DDSP (Fig. 1-4).

* p<0.05 vs. control; **p<0.01 vs. control; *** p<0.001 vs. control; A:B p=0.202; A:C p<0.05; B:C p<0.001, y. =year

Fig. 1. SOD values in different periods of illness in patients with DDSP as compared to the control group.
Fig. 2. Erythrocyte CAT values in different periods of illness in patients with DDSP as compared to the control group * p<0.05 vs. control, A:B p<0.01; A:C p=0.15; B:C p=0.24, y.= year

Fig. 3. GSH-PX values in different periods of illness in patients with DDSP as compared to the control group

*** p<0.001 vs. control A:B p=0.97; A:C p=0.38; B:C p=0.28, y.= year
There was statistically significant difference between the latency and amplitude of sensory evoked potentials (SEP) of medial plantar nerve in the patients and the controls (Tab. 3).

<table>
<thead>
<tr>
<th>SEP n.plantar medialis</th>
<th>Patients mean± SD</th>
<th>Controls mean± SD</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency [ms]</td>
<td>7.50 ± 1.36</td>
<td>5.56 ± 0.89</td>
<td>-8.90</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude [µV]</td>
<td>0.381±0.358</td>
<td>0.849±0.606</td>
<td>5.37</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 3. Latency and amplitude of the SEP of medial plantar nerve in the diabetic neuropathy patients and in the control

All electrophysiological parameters related to the conduction of motor and sensory fibers of the tested upper and lower extremity nerves showed that there was a statistically significant difference between diabetic neuropathy patients and controls (Tab. 4).
### Table 4. ENG parameters in the diabetic neuropathy patients and in the control group.

<table>
<thead>
<tr>
<th>ENG parameters</th>
<th>Controls mean±SD</th>
<th>Patients mean±SD</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Latency (ms)</td>
<td>Latency (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMAP peroneal</td>
<td>3.54±0.56</td>
<td>4.66±1.13</td>
<td>-6.64</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>5.77±2.47</td>
<td>3.46±2.13</td>
<td>5.88</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NCV (m/s)</td>
<td>51.65±5.79</td>
<td>41.24±6.77</td>
<td>9.20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Latency (ms)</td>
<td>Latency (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMAP tibial nerve</td>
<td>3.56±0.51</td>
<td>4.94±1.32</td>
<td>-7.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>11.19±4.73</td>
<td>4.62±2.98</td>
<td>10.28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NCV (m/s)</td>
<td>43.37±3.07</td>
<td>35.72±7.30</td>
<td>7.083</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Latency (ms)</td>
<td>Latency (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMAP median nerve</td>
<td>3.45±0.55</td>
<td>4.88±2.24</td>
<td>-4.47</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>7.00±2.51</td>
<td>4.90±2.35</td>
<td>5.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NCV (m/s)</td>
<td>56.57±7.01</td>
<td>49.52±6.22</td>
<td>6.27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Latency (ms)</td>
<td>Latency (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMAP ulnar nerve</td>
<td>2.90±0.63</td>
<td>3.17±0.78</td>
<td>-2.12</td>
<td>0.0359</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>7.87±3.35</td>
<td>4.93±2.72</td>
<td>6.36</td>
<td>0.0025</td>
</tr>
<tr>
<td>NCV (m/s)</td>
<td>57.54±8.57</td>
<td>48.59±8.00</td>
<td>6.30</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Latency (ms)</td>
<td>Latency (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEP sural nerve</td>
<td>2.93±0.50</td>
<td>4.14±0.90</td>
<td>-8.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>15.61±6.16</td>
<td>8.79±5.77</td>
<td>6.31</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NCV (m/s)</td>
<td>36.37±4.48</td>
<td>29.49±6.35</td>
<td>6.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Latency (ms)</td>
<td>Latency (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEP median nerve</td>
<td>3.96±0.49</td>
<td>4.70±0.82</td>
<td>-5.83</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>43.07±9.33</td>
<td>15.75±9.70</td>
<td>16.47</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NCV (m/s)</td>
<td>47.36±5.63</td>
<td>38.77±7.85</td>
<td>6.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Latency (ms)</td>
<td>Latency (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEP ulnar nerve</td>
<td>3.28±0.68</td>
<td>4.12±0.82</td>
<td>-6.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>36.46±11.63</td>
<td>17.28±12.22</td>
<td>9.16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NCV (m/s)</td>
<td>46.32±6.10</td>
<td>38.20±7.37</td>
<td>6.69</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 4. ENG parameters in the diabetic neuropathy patients and in the control group. CMAP - compound muscle action potential, SEP - sensory evoked potentials, NCV-nerve conduction velocity

A number of scored ENG parameters correlated significantly with erythrocyte SOD and CAT level in the patients studied (Tab. 5 and Tab. 6).

### Table 5. Correlation between SEP parameters of medial plantar nerve and parameters of OS (SOD and CAT) in diabetic neuropathy patients. Marked values point out a statistically significant correlation.

<table>
<thead>
<tr>
<th>SEP of medial plantar nerve</th>
<th>SOD</th>
<th>CAT</th>
<th>GSH-px</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Latency</td>
<td>-0.1621</td>
<td>0.1071</td>
<td>-0.0488</td>
<td>0.6295</td>
</tr>
<tr>
<td>Amplitude</td>
<td>0.0995</td>
<td>0.3248</td>
<td>0.2059</td>
<td><strong>0.0399</strong></td>
</tr>
<tr>
<td></td>
<td>0.0580</td>
<td>0.5564</td>
<td>-0.0570</td>
<td>0.5734</td>
</tr>
</tbody>
</table>

Table 5. Correlation between SEP parameters of medial plantar nerve and parameters of OS (SOD and CAT) in diabetic neuropathy patients. Marked values point out a statistically significant correlation.
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Table 6. Correlation between ENG parameters and parameters of OS (SOD and CAT) in diabetic neuropathy patients. MEP- Motor Evoked Potential, SEP- Sensory Evoked Potentials, NCV – Nerve Conduction Velocity. Marked values point out a statistically significant correlation.

<table>
<thead>
<tr>
<th>Nerve</th>
<th>ENG parameters</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMAP Peroneal nerve</td>
<td>Latency</td>
<td>-0.1096</td>
<td>0.2777</td>
<td>-0.1597</td>
<td>0.1126</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>-0.2526</td>
<td><strong>0.0112</strong></td>
<td>-0.2859</td>
<td><strong>0.0039</strong></td>
</tr>
<tr>
<td></td>
<td>MEP NCV</td>
<td>-0.2633</td>
<td><strong>0.0081</strong></td>
<td>0.0199</td>
<td>0.8442</td>
</tr>
<tr>
<td>CMAP Tibial nerve</td>
<td>Latency</td>
<td>-0.1866</td>
<td>0.0630</td>
<td>0.0406</td>
<td>0.6883</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>-0.1575</td>
<td>0.1175</td>
<td>-0.0568</td>
<td>0.5747</td>
</tr>
<tr>
<td></td>
<td>NCV</td>
<td>-0.2524</td>
<td><strong>0.0113</strong></td>
<td>0.0254</td>
<td>0.8016</td>
</tr>
<tr>
<td>CMAP Median nerve</td>
<td>Latency</td>
<td>-0.2326</td>
<td><strong>0.0199</strong></td>
<td>-0.0959</td>
<td>0.3425</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>-0.0663</td>
<td>0.5123</td>
<td>0.0188</td>
<td>0.8529</td>
</tr>
<tr>
<td></td>
<td>NCV</td>
<td>-0.2171</td>
<td><strong>0.0301</strong></td>
<td>0.0462</td>
<td>0.6483</td>
</tr>
<tr>
<td>CMAP Ulnar nerve</td>
<td>Latency</td>
<td>-0.0442</td>
<td>0.6627</td>
<td>-0.0043</td>
<td>0.9659</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>-0.1531</td>
<td>0.1282</td>
<td>0.0174</td>
<td>0.8639</td>
</tr>
<tr>
<td></td>
<td>NCV</td>
<td>-0.1387</td>
<td>0.1687</td>
<td>-0.0629</td>
<td>0.5339</td>
</tr>
<tr>
<td>SEP Median nerve</td>
<td>Latency</td>
<td>-0.2183</td>
<td><strong>0.0291</strong></td>
<td>-0.0501</td>
<td>0.6205</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>-0.0881</td>
<td>0.3836</td>
<td>-0.0057</td>
<td>0.9549</td>
</tr>
<tr>
<td></td>
<td>NCV</td>
<td>-0.2079</td>
<td><strong>0.0379</strong></td>
<td>-0.0569</td>
<td>0.5741</td>
</tr>
<tr>
<td>SEP Ulnar nerve</td>
<td>Latency</td>
<td>-0.1679</td>
<td>0.0949</td>
<td>-0.1659</td>
<td>0.0991</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>-0.0213</td>
<td>0.8336</td>
<td>-0.0997</td>
<td>0.3239</td>
</tr>
<tr>
<td></td>
<td>NCV</td>
<td>-0.1202</td>
<td>0.2336</td>
<td>-0.0627</td>
<td>0.5357</td>
</tr>
<tr>
<td>SEP Sural nerve</td>
<td>Latency</td>
<td>-0.1129</td>
<td>0.2636</td>
<td>-0.3837</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>-0.2896</td>
<td><strong>0.0035</strong></td>
<td>-0.1449</td>
<td>0.1505</td>
</tr>
<tr>
<td></td>
<td>NCV</td>
<td>-0.1122</td>
<td>0.2662</td>
<td>-0.3840</td>
<td><strong>0.0001</strong></td>
</tr>
</tbody>
</table>

6. Discussion

Results of experimental studies, conducted both in vivo and in vitro, suggest that the peripheral nervous system is sensitive to oxidative damage (Schmeichel et al.,2003; Russel et al., 1999; Russel et al., 2002). Neurons take over glucose from the blood by the concentration dependent transport, so that hyperglycemia is always associated with increased glucose values in the neurons, which results in oxidative stress (Tomlinson and Gardiner, 2008; Andrea et al., 2004). On the other hand, antioxidative defense in peripheral nerves is thought to be limited due to primary lower values of glutathione and glutathione-dependent enzymes (GSH-Px and GSH-r) (Romero et al., 1991; Schmeichel et al., 2003), which further increases the sensitivity of nerves to oxidative damage. SOD could provide efficient antioxidative protection, since, contrary to glutathione-dependent enzymes, it is relatively more active in the peripheral nerves. However, in spite of such a theoretical position, studies carried out on experimental models have not shown any significant changes of the endoneural antioxidative status in the experimentally induced DN, except an increased CAT levels which could not be able to correct by insulin therapy (Van Dam et al., 1996). On the other side, positive effects of antioxidants on the antioxidative capacity of blood, and also on the disturbed function of peripheral nerves in the very same
The determination of OS biomarkers is an important step in the understanding of DN pathogenesis. Recent research suggests that there are tissue- and time-dependent changes in the activity of various antioxidative enzymes. The results of our study show that there is a statistically significant reduction of erythrocyte SOD levels in patients with type 2 DM and DDSP in comparison with healthy controls, which corresponds to the data from the literature (Arai et al., 1987; Kawamura et al., 1992; Vijaylingam et al., 1996). Erythrocyte SOD level was significantly lower in patients with diabetic neuropathy compared with patients without diabetic neuropathy, suggesting the importance of antioxidant protection in the prevention of nerve injury. The main reason for the reduced SOD activity is the glycolization of Cu, Zn-SOD, which has been documented in both in vitro and in vivo experiments (Arai et al., 1987; Kawamura et al., 1992). However, there are also studies which show no changes in erythrocyte SOD activity (Peuchant et al., 1997; Walter et al., 1991; Faure et al., 1995), or which, quite the contrary, suggest an increased activity of this enzyme (Yaqoob et al., 1994).

In most cases, CAT activity in erythrocytes was not changed in either experimental animals or type 1 and type 2 DM patients (Wohaieb & Godi, 1987; Godin et al., 1988; Matkovich et al., 1982). However, some studies have noted changes in CAT activity, in particular its reduction (Vijaylingam et al., 1996; Alphonsus et al., 2007). In this study, erythrocyte CAT values were reduced in patients as compared with the controls, but this reduction did not reach statistically significant levels, which correlates with the literature data listed above. Catalase values were reduced in patients with DSP, compared with patients without DSP, although these deviations were not statistically significant. Reduced CAT activity could be explained by the accumulation of H\textsubscript{2}O\textsubscript{2} in the cells, as a result of glucose autooxidation. As the principal enzymatic role of CAT is to control H\textsubscript{2}O\textsubscript{2} concentration, H\textsubscript{2}O\textsubscript{2} accumulation in the cells is believed to lead to the exhaustion of this enzyme, which primarily reflects erythrocytes, where CAT is otherwise most active. Studies have shown that in other tissues H\textsubscript{2}O\textsubscript{2} accumulation may stimulate CAT synthesis, thus increasing its activity. However, erythrocytes lack the genetic apparatus for such a synthesis, which is a reason that the increased H\textsubscript{2}O\textsubscript{2} concentration results in the exhaustion and inactivation of catalase (Alphonsus et al., 2007).

Changes in the activity of glutathione-dependent enzymes were different in experimental models. Most studies have shown tissue and time dependent changes of enzyme activity. However, even if we acknowledge these factors, we cannot precisely match the results of various studies of the association of DM and activity of glutathione-dependent enzymes. The studies dealing with the GSH-Px activity in the erythrocytes have demonstrated variable results. A large number of studies demonstrated that GSH-Px activity in the blood, erythrocytes, and leukocytes was similar in DM patients (types 1 and 2) and in healthy controls (Walter RM et al., 1991; Leonard M.B. et al., 1995; Akkus I et al., 1996). On the other hand, there are studies clearly confirming altered activity of glutathione-dependent enzymes in DM, above all in the form of reduced activity of GSH-Px and increased activity of GSH reductase (Godin et al., 1988; Dohi et al., 1988; Tagami et al., 1992; Langenstroer & Pieper, 1992; Blaktynty and Harding, 1992). Murakami (1991), studying the erythrocytes in DM, concluded that reduced erythrocyte GSH was caused by reduced activity of gamma glutamyl-cystein synthetase in connection with its glycation, reduced activity of GSH-
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reductase and defective glutathione transport. Yoshida (1995) confirmed that in the erythrocytes of diabetics with poorly controlled disease the synthesis of GSH and thiol transport were damaged, rendering the cells more sensitive to oxidative damage. In diabetics with permanently higher values of glucose Uzel et al. (1987) established reduced GSH-Px activity and lower erythrocyte GSH values, with increased products of lipid peroxidation, with changes more apparent in patients with retinopathy. GSH reduction in the erythrocytes in DM was confirmed by Bono et al. (1987) as well. Stahlberg and Hietanen (1991) observed a reduced activity of GSH reductase in children with DM but Walter et al. (1991) were not able to demonstrate any difference in GSH-Px and GSH reductase activities in diabetics and nondiabetics. Kaji et al. (1985) failed to find a difference in activity of erythrocyte GSH-Px, but established increased GSH-Px activity in the plasma in women with DM. Osterod et al. (1996) found in their studies a reduced erythrocyte GSH-Px activity in DM type 1. Authors of some studies also described an increased erythrocyte GSH-Px activity in DM (Matkovics et al., 1982). Literature data mostly indicated an unaltered or reduced activity of erythrocyte GSH-Px. The results obtained in this study indicate a statistically significant reduction of GSH-Px activity both in patients with DSP and in patients without DSP, compared to controls. Erythrocyte GSH-Px values were significantly lower in patients with diabetic neuropathy compared with patients without diabetic neuropathy. Reduced GSH-Px could be directly explained by reduced GSH content in studied patients, representing otherwise a substrate and cofactor for GSH-Px enzyme.

The studies of glutathione homeostasis in DM have so far shown tissue differences in the levels of GSH, although in most of the cases reduced GSH contents have been found. The studies of glutathione level in the blood of diabetics demonstrated that those with DM type 2 had reduced erythrocyte level of GSH and increased level of GSSG (De Mattia at all, 1994; Jain & McVie, 1994). The blood level of GSH was significantly reduced in various phases of the type 2 DM, such as glucose intolerance, early hyperglycemia (Vijayalingam et al., 1996) in the first two years and before complications developed (Sundaram et al., 1996), as well as in poorly controlled glycemia (Peuchant et al., 1997). Yoshida et al. (1995) observed reduced GSH in the erythrocytes of type 2 diabetics, with disturbed activity of gamma glutamyl transferase and thiol transport. In contrast to a clear reduction of blood GSH in type 2 DM, in type 1 disease the results were not entirely convincing. The role of GSH in the occurrence of diabetes complications has not been elucidated. Thormalley et al. (1996) found an inverse correlation between the level of erythrocyte GSH and presence of diabetes complications (neuropathy, retinopathy, and nephropathy) in patients with DM type 1 and 2, leaving unanswered the question whether GSH levels were reduced in those without complications, and these patients have an even more significant reduction GSH levels.

The results of our investigation showed a statistically significant reduction of erythrocyte GSH level both in patients with DSP and in patients without DSP compared to controls, which was in accordance with the literature data. In the group of patients without DSP, erythrocyte GSH-Px value did not show larger deviations compared to patients with DSP. Some studies of the antioxidative protection system in DM, principally in experimental animal models, were related to the study of impact of disease duration on the activity of antioxidative enzymes. Time-dependent changes of the activity of antioxidative enzymes and prooxidative-antioxidative balance were thus observed in DM in various tissues. (Majythija & Jayesh, 2005; Sasvári and Nyakas, 2003). Kishi et al. (2005) based on their own study, came to the conclusion that changes in the activity of antioxidative enzymes in
peripheral nerves were not the result of reduced gene expression, but can be linked to the duration of DM or posttranslational modifications. Bearing these information in mind, this study analyzed the activity of antioxidative enzymes and glutathione in the blood of patients which disease had lasted up to 5 years, 5-15 years, and over 15 years. The activity of SOD enzyme was generally lower in patients in each phase of the disease, though the reduction was variable. The smallest reduction was observed in the first 5 years of DM, while the reduction was most evident in advanced disease phases - in those affected for more than 15 years. More intense inactivation of SOD in more advanced DM could be explained by increased production of H_2O_2 in the processes of non-enzymatic glycation predominating with time, which can further inhibit SOD. In the group of those affected for 5-15 years, a slight increase of SOD activity can be observed related to other two groups, which can be explained by an adaptive increase of antioxidative protection pathways. Increased H_2O_2 production inhibited SOD activity, but, on the other hand, formation of superoxide radicals presents a signal for increased gene expression of SOD (Matsuyama et al. 1993). Available amount and activity of SOD should represent a balance between the enzyme production and its degradation. As shown here, the balance demonstrated time-dependent modulation in different disease phases.

The activity of erythrocyte catalase also demonstrated a declining tendency, with the smallest decline in the first 5 years of DM. The decline of catalase activity was greatest in those affected for 5-15 years. In this group, the values of erythrocyte catalase were significantly different from control ones.

In all groups, a statistically significant reduction of erythrocyte GSH level was observed compared to controls. Among the groups of different disease phases, there was no statistically significant difference in the erythrocyte GSH level. Changes were observed also with the enzyme associated with glutathione metabolism - GSH-Px. This enzyme demonstrated a statistically significant decline of activity in all disease phases compared to controls. The decline was most evident in advanced DM (>15 years), though there were no statistically significant deviations among the individual groups of patients in different disease phases. Reduced GSH-Px activity can be directly explained by low GSH content in diabetics, in view of the fact that GSH is the substrate and cofactor of GSH-Px. Reduced GSH-Px activity can be caused by the enzyme inactivation as the consequence of increased glycation in the situation of hyperglycemia. Increased glycation and consequential protein reactions can affect amino acids in active enzyme domain or disturb stereochemical configuration and cause structural and functional molecular changes (Bonnefont-Rousselot et al., 2000). Reduced GSH-Px activity causes H_2O_2 accumulation, which also contributes to progressive reduction of activity of SOD in advanced DM. These changes in the activity of antioxidative enzymes in DM confirm the notion that disturbed carbohydrate metabolism have an impact on the function of antioxidative protection which by itself can affect the development of late complications of DM. In recent years, it has been definitely confirmed that the frequency of late diabetic complications is higher with poorer metabolic control. If oxidative stress has a role in the development of diabetic complications, we should expect adaptive changes in the system of antioxidative protection. These changes would be more or less evident in different situations of metabolic control and largely depend on the duration of unfavorable metabolic status.

Similarly to the literature data, our results suggest that blood of type 2 DM patients has decreased antioxidative protection. However, in spite of strong evidence in the literature that OS is increased in DM, there is still no definite connection between OS levels and the
development of late diabetic complications. Accordingly, in this study, we have looked into the interrelation between the tested antioxidative enzyme levels and the functional damage of peripheral nerves. Our previous study did not show any correlation between plasma total antioxidant capacity (TAC) and the degree of damage of peripheral nerves in type 2 DM and DDSP patients (Djordjević et al., 2008). Having in mind that TAC is not a mere sum of various antioxidative activity, but a dynamical system of interdependent individual serum antioxidant parameters (Koracević et al., 2001), we have designed this study in such a way as to observe and analyze the influence of individual TAC constituents on the development of peripheral nerve dysfunction in type 2 DM patients.

All electroneurographic parameters of peripheral nerve conduction have showed deviation in the patients as compared with the control group and these deviations have been statistically significant. In the studied patients, we have found a significant negative linear correlation between erythrocyte SOD levels and a number of scored ENG parameters – indicators of DDSP (the lower the SOD values, the higher the ENG score, i.e. the more pronounced the functional damage). The correlation that was found between SOD and ENG indicators of the degree of neuronal damage indicates that there was an important role of toxic effects of superoxide anion radicals in the development of neuronal damage. In vivo, superoxide anion radicals are mostly removed enzymatically, by SOD. When superoxide anion radicals are excessively produced, they react with nitric oxide and form a peroxynitrite, which has numerous cytotoxic effects. A tenfold increase in superoxide anion radicals and nitric oxide has been found to increase peroxynitrite production one hundred times (Djordjević et al., 2000). Excessive production of superoxide anion radicals, nitric oxide and peroxynitrite may, thus, be a significant pathogenetic factor for neuronal damage.

As for catalase, even though the reduced activity of this enzyme did not reach statistical significance in this study, the correlation analysis between CAT blood levels and the electrophysiological conduction parameters of the peroneal, sural, median, and plantar nerves (MEP-amplitude of peroneal nerve, the latency and NCV of the sural nerve, the SEP amplitude of medial plantar nerve) has revealed a statistically significant value. Such results suggest that CAT has a pathogenetic importance, i.e. that hydrogen peroxide has toxic effects on the degree of neuronal damage.

There was no correlation between erythrocyte GSH-Px and ENG parameters which could be explained by lower Km and lower GSH-Px activity in erythrocytes, compared to CAT activity. Erythrocyte GSH-Px value correlated with sensor nerve conduction of median nerve only. This results could be explained by limited activity of GSH and GSH-dependent enzymes in peripheral nerves. It should also bear in mind that the values of GSH and GSH-px in our group of patients showed small variation over the course of DM, which could also be the explanation for the lack of correlation with the ENG parameters, given the progressive course of diabetic neuropathy.

7. Conclusion

DM is closely linked to an imbalance in pro/antioxidant status of cells and changes in redox potential. Oxidative stress, as a common denominator, is the biochemical mechanism by which disturbed glucose metabolism and deregulation of cell signaling leads to the development of diabetic complications. Results of our study pointed out a reduced systemic antioxidative defense in the patients with type 2 DM and diabetic distal symmetrical polyneuropathy and indicate that systemic oxidative stress plays a potential role in the
development of diabetic neuropathy. For better understanding of the role of oxidative stress and antioxidative mechanisms, further investigations with standardized methodology, molecular biological technique and better defined experimental models and subjects are required, aiming to prevent, delay or slow the progression of the disease.

8. References


The Role of Oxidative Stress in Pathogenesis of Diabetic Neuropathy: Erythrocyte Superoxide Dismutase, Catalase and Glutathione Peroxidase Level in Relation to...


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Role of the Adipocyte in Development of Type 2 Diabetes

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Adipocytes are important in the body for maintaining proper energy balance by storing excess energy as triglycerides. However, efforts of the last decade have identified several molecules that are secreted from adipocytes, such as leptin, which are involved in signaling between tissues and organs. These adipokines are important in overall regulation of energy metabolism and can regulate body composition as well as glucose homeostasis. Excess lipid storage in tissues other than adipose can result in development of diabetes and nonalcoholic fatty liver disease (NAFLD). In this book we review the role of adipocytes in development of insulin resistance, type 2 diabetes and NAFLD. Because type 2 diabetes has been suggested to be a disease of inflammation we included several chapters on the mechanism of inflammation modulating organ injury. Finally, we conclude with a review on exercise and nutrient regulation for the treatment of type 2 diabetes and its co-morbidities.

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