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Influence Of Cocarnit On The Pro- / Antioxidant Balance In Sciatic Nerve Tissue In The Rats With Diabetic Polyneuropathy.

Nikitina NS*, Stepanova LI, Dvorschenko KO, Ostapchenko LI, and Berehovyi SM.

Taras Shevchenko National University of Kyiv, ESC "Institute of biology and medicine", Ukraine. 2/12 Academician Glushkov Avenue, Kyiv 03022, Ukraine.

ABSTRACT

Diabetic polyneuropathy (DP) is a cause of poor quality of life and can cause disability or even death of patients, that increases the relevance of diagnosis and treatment of disease. We investigated the influence of the metabolic drug Cocarnit on the development of oxidative stress with consequent of the pro- and antioxidant imbalance and expression of protein factors, that are an important link in the pathogenesis of DP. Treatment of DP restores pro-antioxidant balance in the nerve and decreased the content of OMP. It's changes the expression of protein factors (decreased the activity of NF-Kb and increased Caspase 3).

Keywords: diabetic polyneuropathy, lipid peroxidation, NF-kB, Caspase -3, Cocarnit

Corresponding author

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INTRODUCTION

Diabetic peripheral polyneuropathy (DP) is a common complication of diabetes [1] and it's a cause of poor quality of life, disability and death of patients, that increases the urgency of diagnosis and treatment of disease. The mechanism of the development of DP is described by various theories, including edema and degeneration of nerve tissues [2]; increased polyol pathway activity leading to sorbitol and fructose accumulation [3], insufficiency of myoinositol [4]; violation of lipid metabolism, increased lipid peroxidation [5]; nonenzymatic glycation of proteins yielding advanced glycation end-products; metabolic stress, that results in increased formation of free radicals [6], activation of the factor NF-kB and increased activity of proteinkinase C.

Hyperglycemia is a trigger for the initiation of oxidative stress [7] (fig.1). Progression of pathophysiological disorders are accompanied of pro- and antioxidant imbalance. First, reactive oxygen species (ROS) attack plasma membrane proteins, that, in turn, are potential lipid peroxidation (LP) stimulators. Increasing ROS production in mitochondria results in the destruction of DNA-strands and activation of poly (adenosinediphosphate-ribose)-polymerase (PARP). PARP activation, in turn, inactivates one of the main enzymes of glycolysis - glyceraldehyde phosphate-dehydrogenase. As a result, glycolysis is blocked at the level of thiophosphates, glucose is directed to the oxidation of the polyol pathway, stimulation of the formation of diacylglycerol and, as a result, proteinkinase C is activate. The process of oxidative modification of proteins (OMB) is consider as one of the possible factors of inactivation of enzymes and a change in the structural organization during oxidative stress. In nerve, these disturbances lead to impaired neural function and loss of neurotrophic support, and long term, can mediate apoptosis of neurons and Schwann cells, the glial cells of the peripheral nervous system [8]. Protecting tissues from the action of ROS during oxidative stress are carried out by enzyme systems. Specific antioxidant enzymes include superoxide dismutase (SOD) and catalase. P.A. Low i K.K. Nickander, who built a model of development DP, noted the increase of free radicals' activity in the sciatic nerve [9].

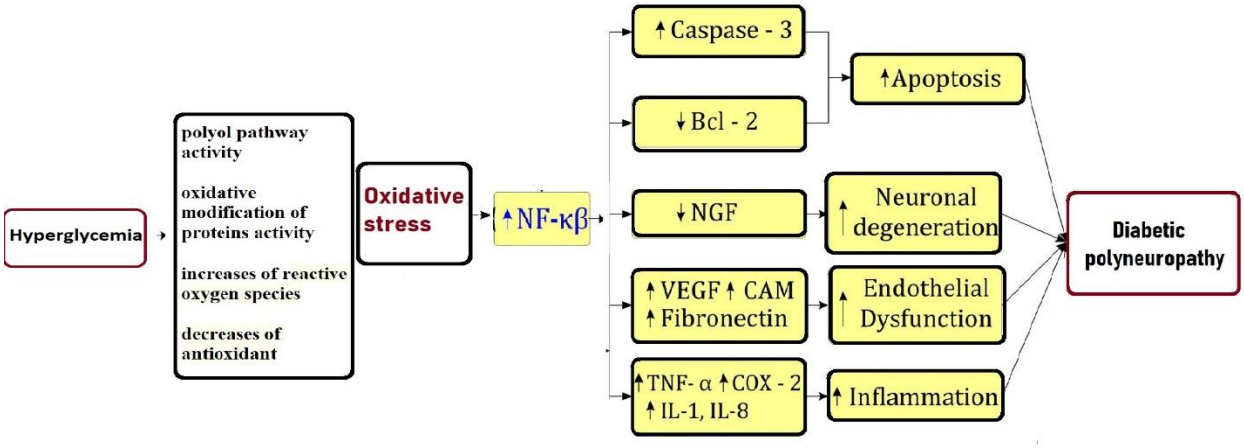

Figure 1: Development of diabetic polyneuropathy

The increase of ROS leads to the activation of NF-kB that important in the pathogenesis complications of diabetes mellitus (fig. 1). Persistent hyperglycemia activates NF-κB that triggers expression of various cytokines, chemokines and cell adhesion molecules. The activation of cyclooxygenase 2, caused by NF-κB, promotes the production of prostaglandin E2 and generation of ROS. Oxidative stress is increasing. NF-κB also regulates the activity of inducible nitric oxide synthase (NO), that is inflammatory enzyme. Cytokines that induced by activation of NF-κB in Schwann cells, endoneuria and epineuria cause infiltration of the nerve by monocytes, follow by its differentiation into macrophages, which contributes to increased proinflammatory processes. As a result, cells are damaged, and myelin are destroyed. Damage of the Schwann cells and the destruction of the neurons are accompanied by a decrease in the ability of the nerve tissue to regenerate

because low level of nerve growth factors. Excessive expression of NF-kB also causes calcification of endothelial cells leading to endothelial dysfunction and subsequent vascular complications [10], leading to leukocyte infiltration and decreased neuronal growth factor, IL6, IL1β, and TNF-α in nerve cell [11]. The increase of ROS can be interdependent with the growth of activity apoptotic processes. Key mechanisms of apoptosis are associate with the functioning of caspase. It's a group of evolutionarily conserved cysteine proteinases that carry proteolysis of proteins, play an essential role in initiating apoptosis. Important factor in cascading apoptotic processes is Caspase -3 [12].

The main advantage in the treatment of DP is provided by means of pathogenetic concept. This is because blood sugar control is not enough to prevent and relieve the symptoms of the disease. Pathogenetic therapy is to prescribe antioxidants and metabolic drugs [13, 14]. Among several drugs our attention attracted a new on our market metabolic drug Cocarnit (World Medicine), that, according to literature, has a positive effect on metabolic, reparative processes, improves trophism of the nervous tissue, has anaesthetizing, vasodilator effect [15]. Cocarnit contains complex of B group vitamins and metabolic substances, that according to our data improves the nervous conductivity [16]. The drug contains 20 mg of nicotinamide, 50 mg of cocarboxylase, 500 mcg of cyanocobalamin, 10 mg of adenosine triphosphate disodium trihydrate (ATP). Nicotinamide (B3) – water-soluble vitamin that has antioxidant action [17]. Nicotinamide is an inhibitor of PARP and a precursor of NAD^+. Thus, it activates glucose oxidation by glycolytic and Pentos phosphate pathways, preventing the progression of dyslipidemia and gluconeogenesis [18]. The positive effect of nicotinamide on restoring sensitivity and nerve conduction, and endothelial function consistent with the key role of activation PARP and oxidative stress in the development of DP. Cocarboxylase is a finished form of coenzyme, that formed from thiamine (B1) in the process of transformation in an organism. Thiamine has been widely used during many decades to treat various neurological diseases of the peripheral nervous system [19]. It has effects on oxidative stress due to increased activity of transketolase - an enzyme that regulates carbohydrate metabolism. It decreases the activity of proteinkinase C, transcription of NF-kB, production of endothelial nitric oxide synthase and endothelin 1. The antioxidant properties of cobalamin (B12) has been demonstrated *in vitro* [20]. It was shown that derivatives of cobalamin inhibit the production of intracellular peroxide, support the intracellular level of glutathione and prevent apoptosis and necrosis. ATP that was previously considered primarily as a necessary component of intracellular biochemical reactions, in recent years has gained new areas. It participates in such processes as perception of pain stimulus, intercellular transmission of excitation in the central and peripheral nervous system, neuroprotective action of endogenous substances, regulation of blood circulation [21].

Thus, oxidative stress is an important mechanism for the development of the DP. However, the influence of Cocarnit on the state of the pro/-antioxidant balance, the content of the transcription factor NF-kB and the cysteine proteinkinase-Caspase-3 are unexplored. Therefore, the purpose of work was to investigate the effect of the drug Cocarnit on treatment of DP in rats.

MATERIALS AND METHODS

All experimental protocols were approved by the Ethical Committee for Conduction of Animal Studies at the Educational and Scientific Center 'Institute of Biology' of Taras Shevchenko National University of Kyiv, Ukraine. Model of diabetic polyneuropathy were carried out on white nonlinear male rats (n=30) which divided into 3 groups. Before the experiment, the rats were kept in quarantine and were marked by given them notches on ears. 1 group – control (healthy rats). Experimental diabetes mellitus type 1 were induced in rats 2 and 3 group by injection a single injection of streptozocin (Sigma, USA) at a dose of 65 mg/kg (i/p) [16]. A glucose level determine with glucometer Free Style Optium XEMV036-P0270 was used and test strips Free Style Optium H were used. Blood was taken from the caudal vein using an intravenous catheter. The tail was washed out, wiped dry, the first drop of blood was wiped, and the second drop was applied to the test strip. A glucose-tolerant test was performed at 30 days to confirm presence of diabetes in rats. The next day, 3th groups of rats were given «Cocarnit» (1 mg/kg, *im*) for 9 days. On the 40th day of the experiment, the rats were sacrificed.

The content of protein in sciatic nerve was measured by the Lowry method [22]. The content of diene conjugates were determined in the heptane-isopropanol extract by spectrophotometric method and the Schiff bases were determined by fluorometric method [23]. The content of TBA-active products was determined by reaction with thiobarbituric acid [24]. An assessment of activity of superoxide dismutase was carried out using

nitrozine tetrazolium [25], for catalase - by reducing the amount of H_2O_2 in solution after incubation under optimal conditions [26].

The content of the transcription factor NF-κB and the cysteine proteinkinase Caspase-3 in homogenate of the sciatic nerve in rats were determined using an enzyme-linked immunosorbent assay ELISA [27]. The content of protein in sciatic nerve was measured Bradford method [28]. Nerve samples were immobilized onto 96-well plate and incubated with corresponding specific primary antibodies (Santa Cruz, USA). After that secondary antibodies conjugated with horseradish peroxidase (Bio-Rad, USA) were added. To enable colorimetric detection, reaction with the substrate o-Phenylenediamine/hydrogen peroxide (Sigma, USA) was performed and absorbance of each well was read at 422 nm. Values were expressed as optical density (OD).

Statistical analysis of data was carried out by the "Statistica 8.0" software package. Shapiro-Wilk's W criterion was used for the investigation of the data distribution type. Posthoc analysis included Student's t-test for parametric data. Whereby differences $P< 0.05$ were deemed reliable [30].

RESULTS AND DISCUSSION

An important non-specific link in the development of various pathological states is the imbalance of oxidative\antioxidant equilibrium and development of oxidative stress [30]. So, the indicators of free radical processes and the state of the antioxidant system can be used as markers for assessing the degree of damage tissues of the body. In our studies, the intensity of free radical processes in the nerve homogenate were evaluated of the content of lipid peroxidation (primary - diene conjugates, secondary - TBA-active products and finite-Schiff bases) and the content of products of oxidative modification of proteins (OMP) of neutral and radical nature with different absorption peaks. The antioxidant system was determined by the activity of antiradical enzymes: superoxide dismutase (SOD) and catalase. By accumulation of diene conjugates can be estimated primary effects of oxidative stress; the content of Schiff bases reflects the efficiency of neutralizing products of free radical oxidation in lipids [31].

Content of diene conjugates is a biochemical indicator of the amount of primary products of lipid peroxidation formed by the use of ROS and free radicals $L^{\cdot}$, $LO^{\cdot}$ and $LOO^{\cdot}$. It was found that in rats with DP content of diene conjugates increases by 42% ($p < 0,05$) in the tissues of nerve compared to the control. In the group of animals with diabetes, which were administered by Cocarnit, the level of diene conjugates in the homogenate of nerve was increases by 25% ($p < 0.05$) compared to the group of animals with diabetes and statistically unsignificantly differ from the level of diene conjugates in control group of rats (table 1).

Table 1: Content of lipid peroxidation products in nerve tissue of rats with diabetic polyneuropathy (M ± m, n=20)

Indicator / Group of rats	Conjugated dienes, nmol × mg of protein $^{-1}$	TBA-active products, nmol × mg of protein $^{-1}$	Schiff bases, conv.unit × mg of protein $^{-1}$
Control	270,28 ± 26,85	402,39 ± 36,59	212,19 ± 18,39
Diabetic polyneuropathy	385,93 ± 34,91*	549,39 ± 50,45*	296,25 ± 25,47*
Diabetic polyneuropathy + Cocarnit	307,36 ± 23,93*, #	453,66 ± 40,04#	258,89 ± 22,38*, #

* – $p < 0,05$– compared to control;
# – $p < 0,05$– compared to group with DP.

In rats with DP content of TBA-active products increased by 36% ($p < 0.05$) compared to control. TBA-active products (malonic dialdehyde and its derivatives) causing modification of proteins and changes in the lipid layer of the membrane. After administration of Cocarnit content of TBA-active products decreased by 21% ($p < 0.05$) compared to rats with DP and statistically unsignificantly differ from control group of rats (table 1).

Due to the oxidative polymerization of modified lipids and proteins and their cross-linking formed Schiff bases, that exhibit fluorescence properties. Content of Schiff bases in the nerve tissue of rats with DP increases by 40% (p <0,05) compared with control group (table. 1). After administration of Cocarnit content of Schiff bases decreased by 14% (p<0.05) compared to rats with DP.

According to literature [32], one of the early indicators of cell damage is the oxidative modification of proteins during oxidative stress (OMP). Inductors of OMP products are reactive oxygen species, an increase in free iron, lipid peroxidation. Free radicals attack proteins throughout the length of the polypeptide chain, breaking the structure that leads to aggregation or fragmentation of the protein molecule [33]. Our studies shown, that content of neutral OMP products in the nerve tissue of rats with DP with absorption peaks of 356 nm and 370 nm were increased by 100% (p <0,05) and 89% (p <0,05), respectively, compared control (table 2).

Table 2: Content of products of oxidative modification of proteins in nerve tissue of rats with diabetic polyneuropathy, conv.unit × mg of protein $^{-1}$, (M ± m, n=20)

Indicator / Group of rats	Neutral character products		Alkaline character products	
	356 nm, aldo-derivatives	370 nm, keto-derivatives	430 nm, aldo-derivatives	530 nm, keto-derivatives
Control	0,037 ± 0,004	0,063 ± 0,006	0,069 ± 0,005	0,032 ± 0,003
Diabetic polyneuropathy	0,074 ± 0,007*	0,119 ± 0,011*	0,101 ± 0,011*	0,055 ± 0,005*
Diabetic polyneuropathy + Cocarnit	0,054 ± 0,005*/#	0,081 ± 0,007#	0,079 ± 0,007#	0,047 ± 0,005*

p < 0,05– compared to control;
– p < 0,05– compared to group with DP.

After injected of Cocarnit these rates were reduced by 37% (p <0,05) and 47% (p <0,05) compared group of rats with DP without treatment. The content of OMP alkaline products with absorption peaks of 430 nm and 530 nm were increased by 46% (p <0,05) and 71% (p <0,05) respectively. After injected of Cocarnit, the level of aldo-derivatives decreased by 28% (p <0,05) relative to the DP (table 2). Thus, in rats with diabetes-induced lesion of nerves Cocarnit recovered content of OMP products of neutral character with an absorption peak at 370 nm and alkaline character with an absorption peak of 430 nm to a control level in the homogenate of nerves. Two other types of OBP products were significantly decreased in group DP+Cocarnit compared to DP rats.

The increase of lipid peroxidation products and the oxidative modification of proteins in the nerves of rats with DP indicates an insufficient antioxidant defense. The activity of antioxidant enzymes in rats with diabetic polyneuropathy in the tissues of nerves were changed. Superoxide dismutase activity increased by 39% (p <0.05), and catalase activity increased by 29% (p <0.05) compared to control. In the group of rats injected Cocarnit SOD were statistically unsignificantly decreased relative DP and increases by 30% (p <0.05) compared to control rats. The catalase activity was increased by 34% (p <0.05) compared to DP and increased by 73% (p <0.01) compared to control (table 3).

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Table 3: Activity of antiradical enzymes proteins in nerve tissue of rats with diabetic polyneuropathy (M ± m, n=20)

Indicator / Group of rats	SOD activity, conv.unit × min^{-1}× mg of protein $^{-1}$	Catalase activity, nmol ×min^{-1}× mg of protein $^{-1}$
Control	0,579 ± 0,053	3,53 ± 0,27
Diabetic polyneuropathy	0,804 ± 0,071*	4,56 ± 0,45*
Diabetic polyneuropathy + Cocarnit	0,753 ± 0,062*	6,11 ± 0,60**/#

* – p < 0,05; ** – p < 0,01 – compared to control;
# – p < 0,05– compared to group with DP.

The increase of ROS results to the activation of NF-kB, that causes expression of various cytokines, chemokines and cell adhesion molecules, which leads to damage of the Schwann cells and destruction of the neurons. The results of our studies shown that content of NF-kB in homogenate of sciatic nerves in rats with DP increased by 28% compared to control (p <0,05) (fig. 2). The NF-kB content in the nerves of rats after treatment decreased by 19% (p <0.05) compared to group with DP.

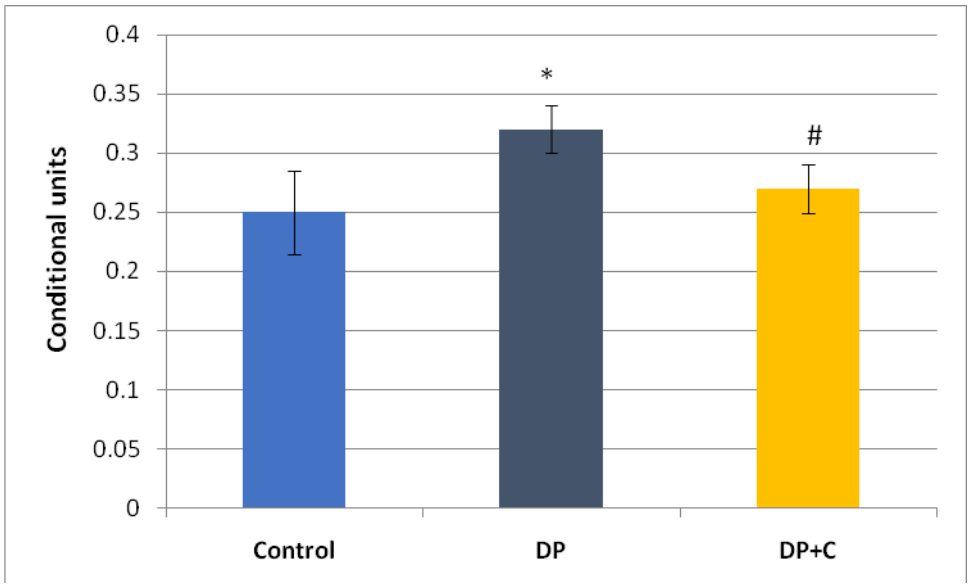

Figure 2: NF-kB content in nerve tissue of control, rats with diabetic polyneuropathy (DP) and after injected of Cocarnit for 9 days (DP + C) (M ± m, n=8).

* – p < 0,05– compared to control;
# – p < 0,05– compared to group with DP.

Content of Caspase-3 protein in nerve of rats with DP increased by 40% (p <0.05) compared control, that is evidence of the occurrence of apoptosis in the nerve tissue (fig. 3). Degeneration of nerve tissue is directly related to the activation of Caspase 3, that is the main effector caspase involved in the apoptosis in Schwann cells [34].

After injected of Cocarnit content of Caspase-3 protein increased by 60% (p <0.05) compared to control group and statistically unsignificantly from the group of rats with DP. It is can related to role of caspase in non-apoptotic processes such as synaptic plasticity [35], involved in neural stem cell differentiation [36].

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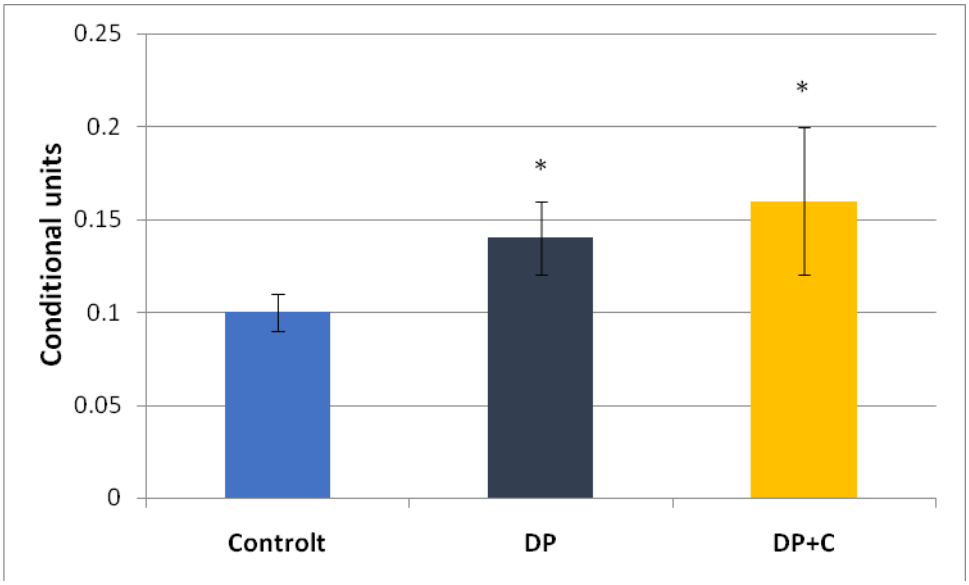

Figure 3: Caspase-3 content in nerve tissue of control, rats with diabetic polyneuropathy (DP) and after injected of Cocarnit for 9 days (DP + C) (M ± m, n=8).

* – p < 0,05– compared to control;

CONCLUSIONS

Thus, it was found that in rats with diabetic polyneuropathy, in response to the intensive formation of free radical products, activation of lipid peroxidation was observed and the content of OMP increased. As a result, the animals were exposed to the activation of the antioxidant system. The LP leads to the activation of NF-Kb, that involve in damage to the integrity of the nerve and Caspase-3, That is a key link in the process of apoptotic processes. Treatment of DP with complex drug Cocarnit restores pro-antioxidant balance in the nerve, decreased the content of OMP. This changes the expression of protein factors (decreased the activity of NF-Kb and increased Caspase 3).

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