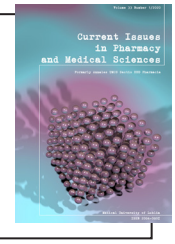
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Influence of the complex drug Cocarnit on the sciatic nerve in the development of diabetic polyneuropathy in rats

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ABSTRACT

Ulcers and slow wound healing are common in diabetic polyneuropathy (DP), as well as shooting or burning pain, sensitivity to touch or lack of sensitivity, low oxygenation of nerve tissue, conductivity disorders and various vascular disorders. The mechanisms of DP development are complex and have not been completely studied. To take into account the role of B group vitamins, we investigated histological structure of nerve tissue, the level of different growth factors and the qualitative composition of active proteolytic enzymes in rats with DP and after the use of the metabolic drug Cocarnit for 9 days. This drug composition include nicotinamide, cocarboxylase, cyanocobalamin, adenosine triphosphate disodium trihydrate. We used an histological study of sciatic nerve; enzyme-linked immunosorbent assay and enzyme electrophoresis methods. In rats with DP, fragmentation of nerve tissue and their necrosis was established. Moreover, degraded forms of plasmin that has a fully functional serine proteinase domain are evident, and, therefore, it exhibits proteolytic properties. DP led to a decrease of neuron growth factor (NGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). After treatment, the histological structure of nerve tissue was significantly improved, and the expression of growth factors NGF and bFGF was increased. Our study demonstrated that administration of Corcarnit brought about the complete restoration of the activation potential of plasmin and the almost disappearance of all degraded forms which were evident in the group with DP.

INTRODUCTION

According to the World Health Organization, diabetic neuropathy is the most common complication of diabetes [1]. Studies suggest that up to 50% of all people with diabetes are affected to some degree. The neuropathy herein can lead to sensory loss and damage of the limbs. Moreover, it is a major cause of impotence in diabetic men. Also, during diabetic polyneuropathy (DP), ulcers and slow wound healing, shooting or burning pain, sensitivity to touch or lack of sensitivity, difficulty moving, trouble with sleeping, increased falls and other effects are indicated [2,3].

For the diabetic, high levels of glucose lead to the formation of different neuronal toxic compounds, such as polyol, sorbitol and fructose [4], abnormal functioning of signal pathways, oxidative stress and mitochondrial dysfunction [5-7]. The reactive oxygen species (ROS) attack plasma membrane proteins, which, in turn, are potential lipid peroxidation 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 the thiophosphates, glucose is directed to the oxidation of the polyol pathway, the formation of diacylglycerol is stimulated and, as an outcome, proteinkinase C is activated [8]. This, however, is only one of the many factors that are involved in the pathogenesis of DP.

In recent years, the spectrum of actions of the plasminogen activator system has extended far beyond blood clot fibrinolysis and restoration of normal blood flow functions [9]. In particular, the plasminogen activator system has been reported to influence several cellular and molecular determinants of inflammation, tissue remodeling and wound healing, making it one of the important mechanisms in the

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regulation of neuroinflammation [10]. In some studies, low oxygenation of nerve tissue due to various vascular disorders is seen in diabetes [11], and during the development of DP, the levels of different protein factors are decreased [12].

Microcirculation of neurons is one of the important factors that are necessary for the normal function of the nervous system. The main factor that is responsible for growth and recovering of blood vessels is VEGF (vascular endothelial growth factor). Some studies have shown that neutralization of Schwann cell-secreted VEGF can be protective.

The course of DP may involve failed signaling from growth factors that act on neurons, but the results of clinical trials using them have been disappointing. A number of neuronal growth factors share downstream signaling cascades that induce survival and growth, but any presumed efficacy in treating DP would depend on whether the involved neurons express relevant receptors [13,14].

Neurotrophic factors can promote the proliferation and differentiation of neural stem cells. One of such important proteins during peripheral diabetic neuropathy is NGF (neurons growth factor), and studies have indicated that combined use of NGF/BDNF (brain-derived neurotrophic factor)/bFGF (basic fibroblast growth factor) promotes proliferation and differentiation of neural stem cells *in vitro* [15]. Moreover, bFGF has effects on the central and peripheral nervous system [16-19]. During DP expression, levels of IGF1 (insulin-like growth factor 1), NGF and bFGF were significantly lower than in a control group [20]. In [21], the expression of bFGF was shown to reduce the apoptosis level of rat Schwann cell line and RSC 96 cells *in vitro*.

Hyperglycemia can cause change of expression of such important factors, as NGF, bFGF and VEGF. These factors play key roles in the recovering of neurons through induction of nerve growth or formation blood vessels, and change of their expression is a possible target for treatment of DP. Nowadays, drugs that reduce the level of sugar, different painkillers, antidepressants are used in treating DP. Practioners also recommend that patients stop smoking, make healthy food choices and be active every day [22,23].

The most promising and affordable areas in the treatment of DP are in utilizing drugs that affect both pathogenetic mechanisms and clinical manifestations of late DP [24]. More and more, the attention of scientific society is focused on vitamins as being compounds necessary for curing different diseases, including polyneuropathy. However, reliable evidence in the literature about the effect of vitamins of the B group are lacking. It was been reported that B vitamins may alleviate some of the symptoms of DP [25], but which combinations of vitamins of the B group, doses and duration of use remain unclear [26].

Vitamin B_{12} occurs in several forms known as cobalamins. Cyanocobalamin is the principal form used in vitamin supplements, while methylcobalamin is a coenzyme form that acts as an important co-factor in the activities of vitamin B_{12} – dependent methyltransferases [27]. Low levels of B_{12} (cyanocobalamin) are a common problem for patients with diabetes, and the therapy of DP by B_{12} showed prominent positive dynamics [28]. Vitamin B_{12} deficiency, which leads to methylcobalamin deficiency, is associated with significant neurological pathology, especially peripheral neuropathy[29,30]. It is also associated with the development of DP. Vitamin B_{12} deficiency may be caused by the use of antidiabetic drugs such as metformin in patients with DP [31,32]. Morphological and histological data also show that long-term use of B_{12} promotes the synthesis and regeneration of myelin in animal models [33]. It was also demonstrated that the use of B_{12} in isolation or in combination therapy in DP led to relief of symptoms and had no effect upon electrophysiology [34].

However, Both Jayabalan and Low describe different experiments with diabetes mellitus neuropathy patients that were administrated methylcobalamin, wherein patients were given vitamin B_{12} for 12-24 weeks [35]. As a result, the investigators suggest that pure methylcobalamin and vitamin B_{12} in isolation therapy are unlikely to be potential candidates for the treatment of DP symptoms.

Our previous studies of biomechanical parameters shown that after treatment for 9 days, cocarnit improves nerve conduction in rats with DP [36]. The drug contains 20 mg of nicotinamide (B3), 50 mg of cocarboxylase (coenzyme of B1), 500 mg of cyanocobalamin (B_{12}) and 10 mg of adenosine triphosphate disodium trihydrate (ATP) [37].

Cyanocobalamin is necessary for hematopoiesis, neural metabolism, DNA and RNA production, and carbohydrate, fat and protein metabolism. B_{12} improves iron functions in the metabolic cycle and assists folic acid in choline synthesis, as B_{12} metabolism is interconnected with that of folic acid. Vitamin B_{12} deficiency causes pernicious anemia, megaloblastic anemia, and neurologic lesions [38].

Cocarboxylase chloride is a form of coenzyme from thiamine in the process of its transformation in the body. It is a required intermediate in the pyruvate dehydrogenase complex and the ketoglutarate dehydrogenase complex, so cocarboxylase is involved in the metabolism of lipids and in the peroxisomal lipid metabolism. It is known that the activity of the ketoglutarate dehydrogenase complex is decreased in many neurodegenerative diseases [39].

Nicotinamide is a critically important part of the structures of NADH and NAD+, where the N-substituted aromatic ring in the oxidized NAD+ form undergoes reduction with hydride attack to form NADH. Niacin and niacinamide have been shown by well-controlled trials to have therapeutic value, the drugs having been used for the management of schizophrenic disorders, drug-induced hallucinations, chronic brain syndrome, hyperkinesis, unipolar depression, motion sickness, alcohol dependence, livedoid vasculitis, acne and leprosy [40,41].

DP is a complex disease that includes many factors of pathogenesis that require detailed study. The aim of present study was to investigate histological structure, expression of NGF, bFGF and VEGF and the total plasminogen activators in rats with DP and after Cocarnit treatment.

MATERIALS AND METHODS

Experimental animals

All experiments were carried out by the standards of the Convention of Bioethics of the Council of Europe in 1997, European Convention for the protection of vertebrate

animals, the general ethical principles of animal experiments approved by first National Congress of Bioethics of Ukraine and other international agreements and national legislation in this field. The model of diabetic polyneuropathy were carried out on white nonlinear male rats (n=30) weighing 200-250 g, which were divided into 3 groups. Before the experiment, the rats were kept in quarantine and were marked by ear notching. The rats were kept in plastic cages (5 rats per box) in a temperature-controlled animal room, with relative humidity 55%, a 12 hour light/dark cycle (light beginning at 7 a.m.) and had free access to feed and water. One group served as control (healthy rats- group 1). Experimental diabetes mellitus type 1 were induced in the rats of 2 and 3 group by single injection of streptozocin (Sigma, USA) at a dose of 65 mg/kg (i/p) [42]. On the 31th day, rats of the 3rd group were given "Cocarnit" (1 mg/kg, i/m) for 9 days. On the 40th day of the experiment, the rats were sacrificed.

Histology

The tissues were embedded in paraffin and a 3-4 micrometer section cut. Paraffin sections were stained with hematoxylin and eosin (H & E). The obtained biopsies of the skin for studies by light-optical microscopy were fixed in 10% solution of neutral formaldehyde and subjected to routine histological treatment [43].

ELISA

The content of the growth factors VEGF, NGF and bFGF in homogenate of the sciatic nerve in rats were determined using an enzyme-linked immunosorbent assay ELISA [44]. The content of protein in sciatic nerve was measured by following the Bradford method [45]. Nerve samples were immobilized onto 96-well plate and incubated with corresponding specific primary antibodies (Santa Cruz, USA). Afterwards, 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.

Enzyme electrophoresis

The applied technique was based upon the method described by Heusen C. and Dowdle E. [46] with modifications by Ostapchenko L. *et al.* [47]. Fibrinogen and plasminogen were incorporated into sodium dodecyl sulfate polyacrylamide gels (SDS-PAG) for plasminogen activators detection. After separation, the gel was incubated in thrombin solution (1 NIH/mL) for 1 h at 37°C. Herein, fibrin formation was required for development of the fibrin-dependent plasminogen activators (t- PA and urokinase) activity [48], and t-PA or urokinase appeared as clear bands corresponding to the area where plasmin has degraded fibrin. The separation gel concentration varied from 11% to 15% to prevent migration of incorporated proteins during electrophoresis. The technique involved: 1) incorporation of fibrinogen or (fibrinogen and plasminogen) into the SDS-PAG of required concentration; 2) electrophoretic separation under usual conditions [36]; 3) gel washing in 2.5% Triton X-100 with shaking for 1 h at 25°C for SDS removal; 4) for (fibrinogen and plasminogen)-incorporated gel - treatment with thrombin solution with shaking for 1h at 37°C; 5) protein visualization according to standard protocol [49]. For electrophoresis and gel staining procedures, the Hoefer Mighty Small system (Amersham Biosciences AB, Sweden) and Hoefer Processor Plus (Amersham Biosciences AB, Sweden) system were used. The sensitivity of the method was ≤0.01 IU of activator or protease per track.

Statistical analysis

Statistical analysis of data was carried out by applying 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. Here, differences $p < 0.05$ were deemed reliable [50].

RESULTS

In the group of control animals, the nerve trunks in cross and longitudinal sections were patterned conventional histological structures (Fig. 1A). Externally, the trunk of the peripheral nerve was found to be coated with an epineurium. The epineuria consisted of fibrous structures in which there are fibroblasts, macrophages and adipocytes. From the epineurium, the nerve leave partitions (perineuria) divided the trunk of the peripheral nerve into separate bundles of nerve tissue. The perineum was comprised of longitudinally oriented thin collagen and elastic tissue and connective tissue cells. Inside the individual beams of nerve tissue, there was also connective tissue called an endoneurium. Nervous tissues are tight; clearly visible processes of neurons and nuclei of neurolemocytes were indicated.

In the group of rats with DP (streptozocin-induced) without treatment, fragmentation of nerve tissue and their necrosis were established (Fig. 1B), this effect is the outcome of dystrophic-degenerative changes in the nerve tissue. The nerve tissues in the transverse section were defined as separate groups, among which, different sizes of voids were seen. These were formed as a result of necrosis of a part of the nerve tissue. The obtained results correlates with data of another group of scientist [51], therefore, the obtained images demonstrate DP development.

In Fig. 1B, dystrophic-degenerative changes in the nerve trunk are noticeable. These include fibrinous-erythrocyte blood clots in the lumen of the arteries and veins, whose walls are moderately thickened, and small areas of necrosis of muscle tissue (along the lower edge of the drawing, areas more intensely perceiving the dye).

In the group of rats with DP (streptozocin-induced) that were treated with Cocarnit, changes were observed in the nerve trunks, but they were much less pronounced than in the group without treatment (Fig. 1C). Moreover, only small areas of dystrophic-degenerative changes (in the form of small slit-like cavities in a transverse section of the nerve) were noted in the nerve trunks, but in general, the structure of the nerve was preserved.

In the longitudinal sections of the nerve, there is only a certain tortuosity of the nerve tissue and small patches of degenerative changes that are visible in the nerve tissue at a high magnification of microscope. There are practically

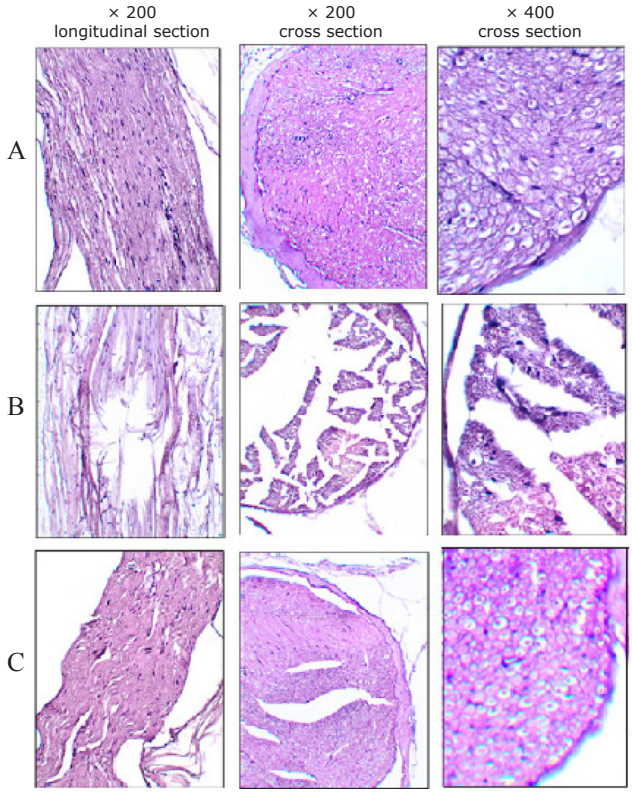
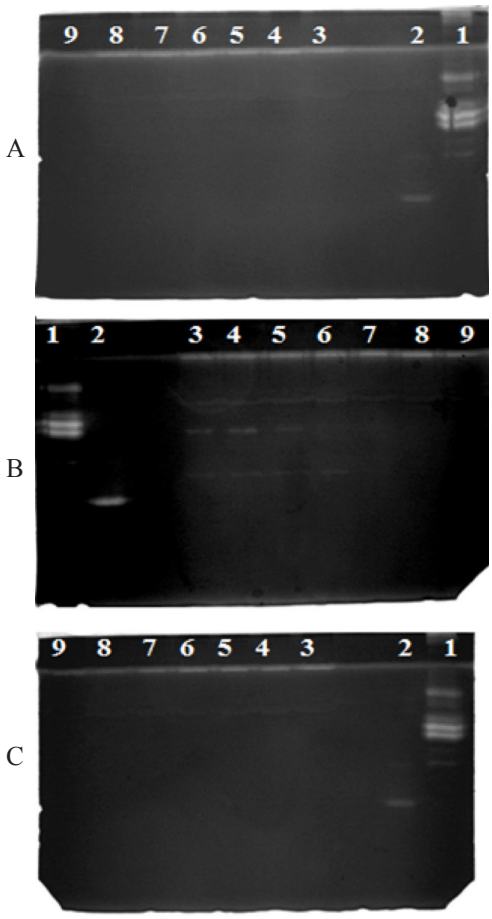


Figure 1. Histological sections of sciatic nerve of control (A), rats with diabetic polyneuropathy (B) and rats with diabetic polyneuropathy after Cocarnit (1 mg/kg) treatment for 9 days (C). Coloring with hematoxylin-eosin



kDa	Intact	DP	DP+C
85	+	+	+
39	-	+	traces
36	-	+	-
30	-	+	-

Figure 2. Enzyme electrophoregram of sciatic nerve of control (A), rats with diabetic polyneuropathy (B) and rats with diabetic polyneuropathy after Cocarnit (1 mg/kg) treatment for 9 days (C): 1 – plasmin standard; 2 – trypsin standard; 3-9 – respectively, samples (n=7)

no changes in the nerves presented in the areas of the muscles with the congestive nerve bundles, which can seen in both transverse and longitudinal sections. Thus, treatment of rats with DP using the metabolic drug Cocarnit led to almost complete restoration of nerve structure.

There are a lot of described mechanisms of diabetic polyneuropathy [52,53]. However, some mechanisms are still unknown, for example, the qualitative composition of active proteolytic enzymes. In our experiment, the total plasminogen activators were determined using enzyme electrophoresis (Fig. 2). Here, plasminogen was prepared in the presence of both fibrinogen and Glu-plasminogen, and the activity of plasminogen activators was visualized as bands cleared from fibrin by the activated proenzyme included to the gel.

Our work indicated that the qualitative composition of active proteolytic enzymes have substrate specificity toward to fibrinogen. Our results show the presence in the sciatic nerve of control rats of only one active zone that contains a protein with a molecular weight of 85 kDa. According to the literature, this is a plasmin [54]. In the group with DP we saw several active zones with lower molecular weights than plasmin. We can assume that these are degraded forms of plasmin that have a fully functional serine proteinase domain and therefore are able to exhibit proteolytic properties. In the group of rats with DP after Cocarnit treatment for 9 days, complete restoration of the activation potential of plasmin and the almost disappearance of all degraded forms was noted.

Still, the mechanisms of action Cocarnit on the curing of diabetic polyneuropathy is unclear. So, we purposed, that this drug has influence on the expression of different growth factors, such as NGF, VEGF and bFGF that are involved in neuron regeneration [55].

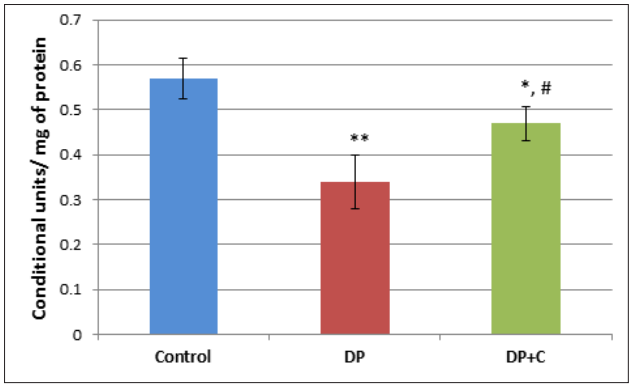
During diabetic polyneuropathy, the level of NGF in the sciatic nerve of rats decreased by 40% ($p < 0.01$) in comparison to that of the control animals. Treatment of Cocarnit led to increasing this figure by 27% ($p < 0.05$) as compared with the group of rats with diabetic polyneuropathy. The outcome, however, did not reach control values (Fig. 3).

One of the most important factors during healing of polyneuropathy is normal oxygenation and transport of nutrients to the damaged place. These processes are possible only if blood vessels are covering the problem place. In normal situations, our nerves do not need such amount of oxygen and nutrients, but during pathology this amount should be increased. This becomes possible only if vasculogenesis is activated, and one of the main factors that can start this process is VEGF.

The expression of VEGF in the sciatic nerve of rats with diabetic polyneuropathy is evident in Figure 4. As indicated, the level of VEGF decreased by 42% ($p < 0.01$) on

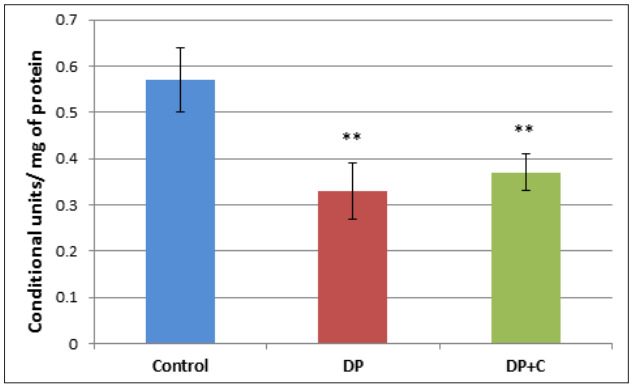
comparison with the control group of animals. Administration of Cocarnit during 9 days did not lead to any changes in comparison with group of rats with DP and was also decreased relative to the control group.

Another growth factor that can be neuroprotective is bFGF. Our experiments demonstrated decreased levels of bFGF in the sciatic nerve of rats with DP by 44% ($p < 0.01$). After Cocarnit treatment, bFGF increased by 30% ($p < 0.05$) (Fig. 5).



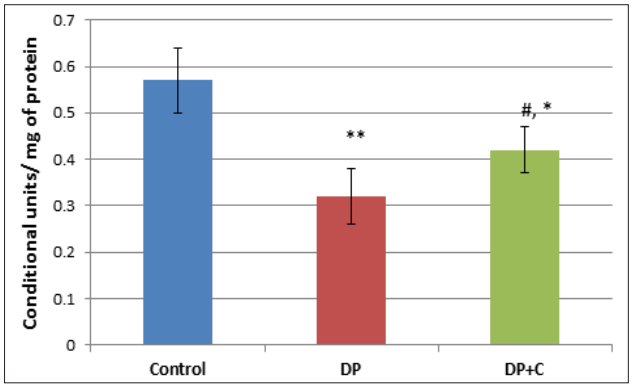
* – p<0,05, ** – p<0,01 when compared with the control group,
# – p<0,05 when compared with rats with diabetic polyneuropathy

Figure 3. NGF content in nerve tissue of control, rats with diabetic polyneuropathy (DP) and rats with diabetic polyneuropathy after Cocarnit (DP + C) (1 mg/kg) treatment for 9 days (M ± SD, n=10)



** – p<0.01 – as compared to control group

Figure 4. VEGF content in nerve tissue of control, rats with diabetic polyneuropathy (DP) and rats with diabetic polyneuropathy after Cocarnit (DP + C) (1 mg/kg) treatment for 9 days (M ± SD, n=10)



* – p<0.05, ** – p<0.01 – as compared to control group
# – p<0.05 – as compared to rats with diabetic polyneuropathy

Figure 5. bFGF content in nerve tissue of control, rats with diabetic polyneuropathy and (DP) and rats with diabetic polyneuropathy after Cocarnit (DP + C) (1 mg/kg) treatment for 9 days (M ± SD, n=10)

DISCUSSION

Diabetes mellitus type 1 in rats was diagnosed on the 28th day after administration of streptozocin. This was demonstrated by persistent hyperglycemia and the glucose-tolerant test. These results are presented in our previous articles [56]. Also in these rats, diabetic polyneuropathy was registered by biomechanical analysis [57]. However, we believe it is also important to conduct histological studies to confirm the development of diabetic polyneuropathy. Histological study of the sciatic nerve in rats with diabetic polyneuropathy showed pronounced dystrophic-degenerative changes – fragmentation of nerve tissue, their necrosis with the formation of cavities. After injection with Cocarnit small areas of dystrophic-degenerative changes in the form of small slit-like cavities in the sciatic nerve tissue were evident, but, in general, the structure of the nerve was restored. However, a detailed study of the pathology of DP requires the determination of structural changes in the nervous tissue in patients with diabetes. Studies of peripheral nerves in humans are limited due to the invasive nature of the biopsy. Cross-sectional studies of patients with diabetes of varying severity and type of neuropathy have shown a range of pathologies characterized by demyelination and axonal loss, as well as microangiopathy [58]. Other researchers have demonstrated structural changes in the peripheral nerve of rats with streptozocin-induced diabetes [59]. Further studies have revealed a decrease the amount of nerve tissue, as well as demyelination. Such effects can be detected 1 month after the onset of diabetes and suggest the presence of DP in rats.

Enzyme electrophoregrams of thesciatic nerve in rats with DP showed the appearance of several active zones with lower molecular weight than the plasmin. Decrease of their molecular weights occurred by removing the curl structure as a result of the action of some enzymes or the process of autolysis of the molecule. The existence of such active degraded forms of plasmin is a factor of the risk of uncontrolled protein-protein interactions. It can lead to the appearance of various fragments of proteins and active peptides. Moreover, the appearance of such degraded forms of plasmin indicates a certain hyperactivation of the plasminogen system as a result of the launching of certain processes associated with the development of the pathological condition. After Cocarnit treatment, we showed positive effects on the processes preceding the formation of degraded forms of plasmin in the nerve.

Our study indicated that the level of NGF in the sciatic nerve of rats with DP had decreased. Previous research established that expression of NGF during diabetic polyneuropathy is dramatically reduced. In addition, many studies have demonstrated that during DP, growth factor deficiencies come about due to decreased synthesis, or function, e.g. an inability to bind to their receptor, and/or abnormalities in nerve transport and processing [20,60].

Although it is reasonable to conclude that diabetic polyneuropathy can be cured by applying exogenous NGF, clinical trials involving the administration of recombinant human NGF have had questionable results. On phase I and II of such trials, results were positive, with positive clinical signs of patients with DP, but during these trials, there was

no proof of nerve tissue recovery. On phase III, immunohistochemistry and quantification of nerve fiber density were conducted using the pan-neuronal marker PGP 9.5 and the results were definite. Exogenous NGF did not have an effect on nerves and all positive outcomes were related to theh indirect influence on blood vessels recovery [61-63].

Many growth factors have been suggested as useful treatments for preventing neurodegeneration, including the vascular endothelial growth factor (VEGF) family. We showed decreased levels of VEGF in the homogenate of nerve tissue of rats with DP. Prior studies have established that vascular endothelial growth factor-A prevents diabetic neuropathic pain and sensory neuronal degeneration in rats with diabetes type I, possibly through an effect on TRPA1 activity [64]. Such study saw that the serum VEGF level was increased in patients with DP, particularly in patients in the neurologically active 'symptomatic' stage [65]. Another study [12] demonstrated that hyperglycemia early affected neurite outgrowth through the impairment of Schwann cells-derived VEGF/FLT-1 signaling and that the neutralization of Schwann cells-secreted VEGF was protective both *in vitro* and in vivo models of diabetic neuropathy.

Recent research in molecular biology show that bFGF is a multifunctional growth factor that stimulates angiogenesis, acts as a vasodilatation factor [66,67], has antiapoptotic effects [68], and induces proliferation in various kinds of cells [69]. The effects of bFGF have been investigated in the field of wound healing [20], bone regeneration, acute ischemic models, and myocardial infarction, both experimentally and clinically [17,70-72]. But experiments with diabetic neuropathy in rats with intravenous injection of bFGF in different doses had uneven outcomes - sciatic nerve blood flow was found to increase, but motor nerve conduction velocity did not change. Meanwhile, continuous intramuscular injection of endogenous in low doses of bFGF led to recovering of motor nerve conduction in patients with diabetic polyneuropathy [73]. Neural cell degeneration and decreased nerve blood flow have been recognized as pathophysiological characteristic features of diabetic neuropathy. Therefore, agents that can act as both as neurotrophic and angiogenic factors may be useful for treatment of diabetic neuropathy, even at an advanced stage [74,75]. Hence, the decreased level of bFGF in our experiments can be explained by their acting on neuron recovering and by the angiogenesis of other factors.

Our results suggest that vitamins of B group have positive effect on the treatment of DP. Thus the cocktail of B vitamins may prove to be a potentially inexpensive and safe long-term approach for treating diabetic neuropathy. We suggest that diverse compositions of vitamins B group can be a key to the healing of diabetic polyneuropathy.

CONCLUSIONS

1. Fragmentation of nerve tissue and their necrosis in rats with diabetic neuropathy are indicative of dystrophic-degenerative changes in nerve tissue;
2. Degraded forms of plasmin have a fully functional serine proteinase domain and therefore are able to exhibit proteolytic properties;
3. Decrease of expression of growth factors NGF, VEGF and bFGF in rats with DP can be mitigated by treatment of rats with DP with the metabolic drug Cocarnit. In our study, this led to almost complete restoration of nerves structure and the complete restoration of the activation potential of plasmin and the almost disappearance of all degraded forms. Herein, cocarnit treatment brought about an increase of NGF and bFGF expression.

CONFLICT OF INTERESTS

Authors declare that they have no conflict of interests

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