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CHANGES IN LIPID COMPOSITION AND FUNCTIONAL ACTIVITY OF DAMAGED SOMATIC NERVES UNDER THE ACTION OF FISETIN AND INSULIN-LIKE GROWTH FACTOR-1

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Annotation. The lipid composition and phospholipase activity of damaged somatic nerves against the background of fisetin and insulin-like growth factor-1 (IGF-1) action were investigated. It was shown that nerve transection is accompanied by an increase in the content of phosphatidylinositol (PI) and free fatty acids (FFA) and a decrease in the level of diacylglycerol (DAG) in both proximal and distal sections of the nerve conductor, apparently as a result of inactivation of phosphoinositide-specific phospholipase C. Against the background of intramuscular administration of IGF-1 at a concentration of 100 ng/kg, intensification of phosphoinositide metabolism, accumulation of DAG and reduction of FFA levels were observed. When fisetin was administered, insignificant changes in the content of FI and DAG were observed throughout the entire period of the experiment in both proximal and distal sections of the nerve conduit. The methods of Raman spectroscopy and action potential registration revealed the restoration of the physicochemical state of the lipid bilayer and functional activity in the proximal segment of somatic nerves when these drugs were used; nevertheless, the mechanism of their action remains different. We believe that IGF-1 administration intensifies the metabolism of phosphoinositide cycle components, indicating a phospholipase C - mediated mechanism of IGF-1 action in injured somatic nerves, which further leads to the formation of components of the phosphatidylinositol-3-kinase signalling pathway involved in the stimulation of expression of various transcription factors necessary for axonal regeneration and restoration of the functioning of injured nerve conductors. In contrast to IGF-1, the absence of intensification of phosphoinositide metabolism and increased synthesis of protein fraction under fisetin action indicates acceleration of regeneration processes in the injured nerve conductor, most likely caused by activation of mitogen-activated protein kinase signalling pathway, which is accompanied by expression of genes associated with remyelination and restoration of physicochemical state of nerve fibre membrane due to protein synthesis.

Keywords: phosphoinositides, diacylglycerol, free fatty acids, fisetin, insulin-like growth factor-1, regeneration, peripheral nervous system.

Introduction

Currently, pharmacological correction of various pathologies of the peripheral nervous system (PNS) is a significant social problem due to the low degree of recovery of normal somatic nerve function after injury and long treatment periods [1]. In this regard, in recent years there has been an active search for various ways to stimulate axonal regeneration, in particular, the study of the role of various substances of biological origin in the regulation of intracellular signalling pathways necessary for the intensification of regeneration processes in injured nerve conductors [2, 3]. According to available literature data, it is known that flavonoids can participate in the processes of gene expression, change the activity of regulatory proteins and participate in the regulation of cell division [4, 5]. Fisetin is one of the promising substances of this group, showing

antioxidant, antiangiogenic, anti-inflammatory, neuroprotective and anti-apoptotic properties [6, 7, 8, 9]. Nevertheless, the molecular mechanisms of activation of the processes of somatic nerves functioning recovery after trauma on the background of its action remain unexplored. In addition, the mechanisms of action of various neurotrophic factors on the regeneration of damaged nerve fibres remain unexplored to date. In the literature there is more and more data indicating the participation of growth factors, in particular, IGF-1 in the regulation of proliferation and differentiation of nerve cells [10]. It is known that the membrane of nerve cells is a multicomponent system in which structural organisation and functions are interrelated, and their changes are the main mechanism of cell transition from one metabolic state to another. Lipids largely determine the development of pathological processes, since they are characterised by high lability, and the products of lipid metabolism cause destructive changes in the membrane. A significant regulatory role is assigned to one of the most labile components of the lipid phase, namely, phosphoinositides, during the metabolism of which secondary mediators are formed that affect the activity of protein kinases and calcium ion transport [11]. In addition, it was shown in earlier studies that degeneration processes in the nerve conductor are accompanied by changes in the lipid composition, which affects the microviscosity of the lipid bilayer and the functional state of the nerve fibre [12].

Based on the above, the aim of this work was to study the effect of fisetin and insulin-like growth factor-1 on changes in lipid composition and membrane state of somatic nerves during injury.

In order to achieve the objective of the paper, the following objectives were set:

- To investigate the effect of fisetin and IGF-1 on the changes in FI, DAG and SFA content in proximal and distal sections of the sciatic nerve after transection;

- To study changes in the state of rat somatic nerve membranes and their functional activity under the action of fisetin and IGF-1, and to establish their role in the regulation of regeneration processes in damaged nerve conductors.

Materials and methods

The experiments were performed on sexually mature male Wistar rats weighing 250-350 g, kept in vivarium conditions on a standard diet without food and water restriction with a change of light regime every 12 hours. The object of the study was the sciatic nerves of rats. In animals of the first experimental group the sciatic nerve was transected at the level of the middle of the thigh. Animals of the second group after transection were injected daily intramuscularly with *fisetin* (*Fisetin*, SIGMA-ALDRICH, Saint Louis, Missouri, USA) in concentrations of 25.0 and 50.0 mg/kg. Animals of the third experimental group were injected intramuscularly daily after transection with *insulin-like* growth factor-1 (*Recombinant Human Insulin-like growth factor type1 (E. coli*), SIGMA-ALDRICH, Saint Louis, Missouri, USA) at concentrations of 75 and 100 ng/kg. The proximal and distal ends of the nerves were extracted after 7, 14, 21 and 28 days and placed in Ringer's solution. Intact animals served as controls. Extraction of lipids from nerve tissue was performed according to the Blye-Dyer method [13]. Phospholipids were separated by one-dimensional thin-layer chromatography on silica gel in the solvent system chloroform/methanol/water/ammonia (60/34/4/2) [14]. The heptane/diethyl ether/ glacial acetic acid system (60:40:1 by volume) was used to separate DAG and SLC [15]. To visualise individual lipid fractions, a reagent for plate staining was prepared beforehand: 20 g of copper sulphate pentahydrate was dissolved in 200 ml of distilled water, 8 ml of sulphuric acid (98 %) and 8 ml of orthophosphoric acid (85 %) were added. After separation in the solvent system, the plate was

placed in the dye for 15 seconds and air dried, after which it was heated on a hotplate at 140 °C for 30 minutes and brown staining of the lipid fractions was observed [16]. Individual phospholipid fractions were identified using Rf values, specific staining agents and standards. Quantitative determination of phospholipids was carried out by densitometric method on the automated CAMAG TLC Scanner 4 complex (Switzerland). Changes in the physicochemical state of the lipid bilayer of nerve fibres were recorded using Raman spectroscopy on a Raman spectrometer in via Basis by Renishaw (England). Bioelectrical activity was recorded for an isolated nerve at its extracellular withdrawal with the following stimulation parameters: amplitude 1.5 V, duration 0.3 ms, stimulation frequency 100 imp/s [17]. Stimulation was carried out by electrical rectangular pulses from the ESL-2 stimulator. The action potential was observed on the screen of the oscilloscope GW Instek GDS-71042. Statistical processing of the obtained data was performed on the basis of ANOVA analysis of variance. Comparison of experimental variants was carried out at 5% significance level.

Results and their

It is known that the physiological status of nerve cells and their functional state are determined by the composition and content of cell membrane lipids [18]. The most labile lipid fraction, phosphatidylinositol, plays a primary role in the processes of transport and regulation of the activity of protein signalling complexes; its phosphorylation results in the formation of its derivatives, phosphoinositides, which are precursors of the most important secondary messengers, inositol triphosphate and diacylglycerol, taking part in the release of calcium ions from intracellular depots and activation of protein kinases [19]. On this basis, at the first stage of the experiment, we studied the changes in the content of phosphatidylinositol, diacylglycerol, and free fatty acids in the proximal and distal sections of the sciatic nerve after its transection and the action of various physiological active substances.

The study revealed that nerve transection is accompanied by an increase in the concentration of phosphatidylinositol in the proximal segment, the maximum accumulation of which is noted on the seventh day of the experiment: in this variant of the experiment the excess over the control level is 76.8 %. With the increase of postoperative terms up to 28 days, the FI content decreases, but still exceeds the control value by 1.3 times (Fig. 1).

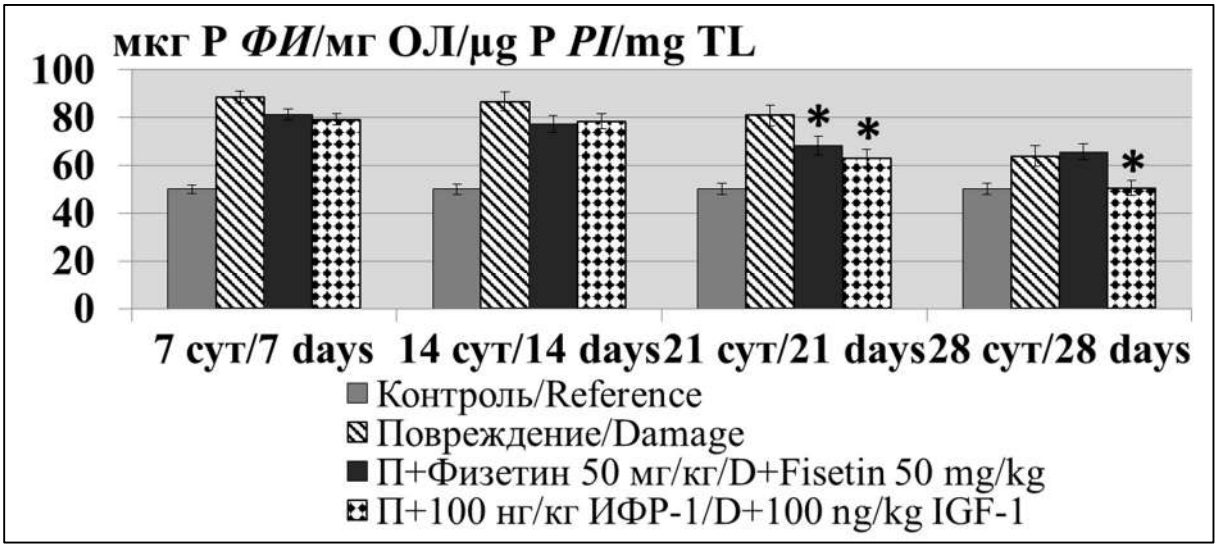

Figure 1. Effect of fisetin and IGF-1 on changes in phosphatidylinositol content in the proximal end of the rat sciatic nerve after transection: µg P *FI/mg* OL - µg inorganic phosphorus phosphatidylinositol/mg total lipids (all error bars indicate the standard error of the mean (n = 10), *- reliability of the difference with respect to the lesion, $p<0.05$)

In the distal part of the nerve conduit there is a more pronounced increase of the studied index on average more than 2.0 times compared to the control by the 7th day of observation. After 21 and 28 days the FI content exceeds the control value by 76.7 and 57 %, respectively. The obtained data indicate that the disappearance of central innervation in the distal part of the nerve conduit after its transection is accompanied by a pronounced intensification of oxidative processes [20].

Currently, one of the topical areas of regenerative biomedicine is the study of the role of various signalling pathways involving physiologically active substances in the processes of healing and restoration of neural tissue functioning. Studies in recent years have shown that derivatives of some flavonoids can also be successfully used in the treatment of various diseases, as they have less toxicity and do not show side effects compared to similar drugs [6, 7]. Plant polyphenolic compounds are able to influence the functioning of cytokine receptors, tyrosine kinase receptors, G-protein coupled receptors and a wide class of transmembrane protein signal transporters [8, 9]. Nevertheless, the molecular mechanisms of these effects are poorly understood. On this basis, in the next series of experiment we investigated the effect of fisetin, a substance of polyphenolic nature, on the change of phosphatidylinositol content in the rat sciatic nerve after its transection. It was shown that in the proximal segment of the nerve the most pronounced change in the FI level was observed on the 21st day of the experiment at a drug concentration of 50 mg/kg, which was accompanied by a decrease in its content by an average of 15.7 % compared to the damage (Fig.1).

There is increasing evidence in the literature that insulin-like growth factor-1, a neurotrophic factor, plays an important role in neuronal development, repair after injury and neurite outgrowth after nerve fibre injury. Studies have shown that IGF-1 is secreted by Schwann cells in an autocrine manner after peripheral nerve injury and promotes more efficient proliferation and myelination of injured nerve cells [21]. In a series of experiments with the administration of

insulin-like growth factor-1 it was shown that the most pronounced changes in the FI content are observed against the background of the drug action at a concentration of 100 ng/kg on the 21st day of the experiment in the proximal segment of the nerve, which is accompanied by a decrease in its level by an average of 21.9% compared to the damage, and on the 28th day, as a result of which the FI level reaches control values (Fig.1). In the distal part of the nerve, the effect of fisetin and IGF-1 is less pronounced compared to its proximal part and practically does not differ from the experiment with damage throughout the entire period of the experiment.

It is known from the literature that diacylglycerol is a degradation product of phospholipase C and plays an important role in Ca^{2+} transport. In addition, DAG has the ability to activate protein kinase C, which is involved in enhancing the signal from the excited cell surface receptor to executor proteins and phospholipase A_2, and acts as a source of arachidonic acid [22]. The amount of DAG in intact nerves has been found to average 14 μg LC DAG/mg total lipids (OL). Nerve transection is accompanied by a decrease in its level. At the same time, the minimum DAG content is observed on the 7th day: in this variant of the experiment the concentration of DAG decreases relative to the control by 63.1 % and 93.5 % in the proximal and distal sections of the damaged nerve, respectively. With the increase of time after transection up to 28 days, an increase in the level of DAG is noted, but it is still below the control value on average by 1.6 times in both segments of the nerve conduit. The experiment showed that the most pronounced changes in the DAG content in the proximal segment of the nerve are observed when fisetin is administered at a concentration of 50 mg/kg and accompanied by an increase in its level by an average of 24.3 % and 11.1 % by the 21st and 28th days after transection, respectively, compared to the damage. In a series of experiments with the administration of insulin-like growth factor-1, there is an increase in the level of DAG by an average of 15.4 %, 73.4 % and 47.1 % by the 14th, 21st and 28th days after transection, respectively, compared to the damage (Fig. 2).

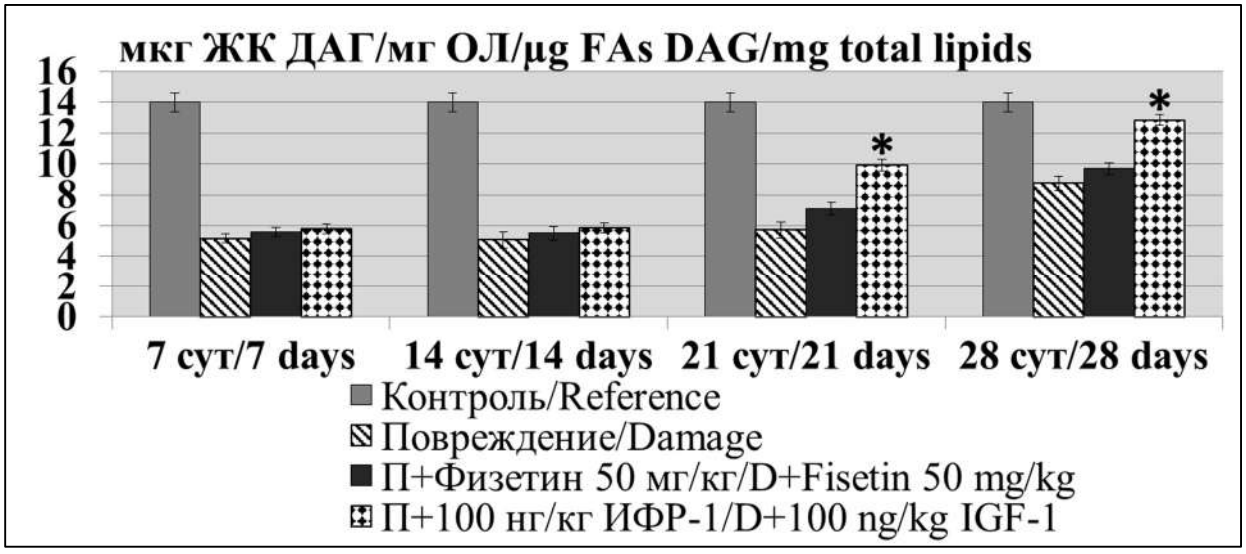

Fig. 2 - Effect of fisetin and IGF-1 on the change of diacylglycerol content in the proximal part of the rat sciatic nerve after its injury: μg LC DAG/mg OL - μg diacylglycerol fatty acids/mg total lipids (all error bars indicate the standard error of the mean (n = 10), *- reliability of the difference in relation to the injury, $p<0.05$)

In the distal part of the nerve conductor, a slight increase in the DAG level by the 28th day of the experiment is observed against the background of intramuscular injection of fisetin in its maximum concentration, but the DAG content in this variant of the experiment is still lower than the control value by 48.4 %. The effect of IGF-1 in the distal part of the nerve is also less pronounced compared to its proximal part and does not cause reliable changes in the studied parameters.

The obtained data on the study of FI and DAG content correlate with changes in the level of free fatty acids. Free fatty acids are known to regulate the work of ion channels and ATPases, G-proteins, protein kinases, modulate phosphoinositide and sphingomyelin cycle, acting as secondary messengers and mediating the effects of many other bioactive molecules [23]. According to the results of our studies, the content of SFAs in the nerves of the control group of animals averages 20.56 μg LC/mg OL. At nerve transection significant changes in the level of free fatty acids in the proximal part of the nerve are observed: their maximum accumulation occurs on the 7th day of the experiment and amounts to 51.76±2.44 μg LC/mg OL. The increase in the duration of the period after the injury up to 28 days is accompanied by a decrease in the amount of FFAs, nevertheless, the level of this index is still significantly different from the control one, exceeding it on average by 1.7 times (Fig. 3).

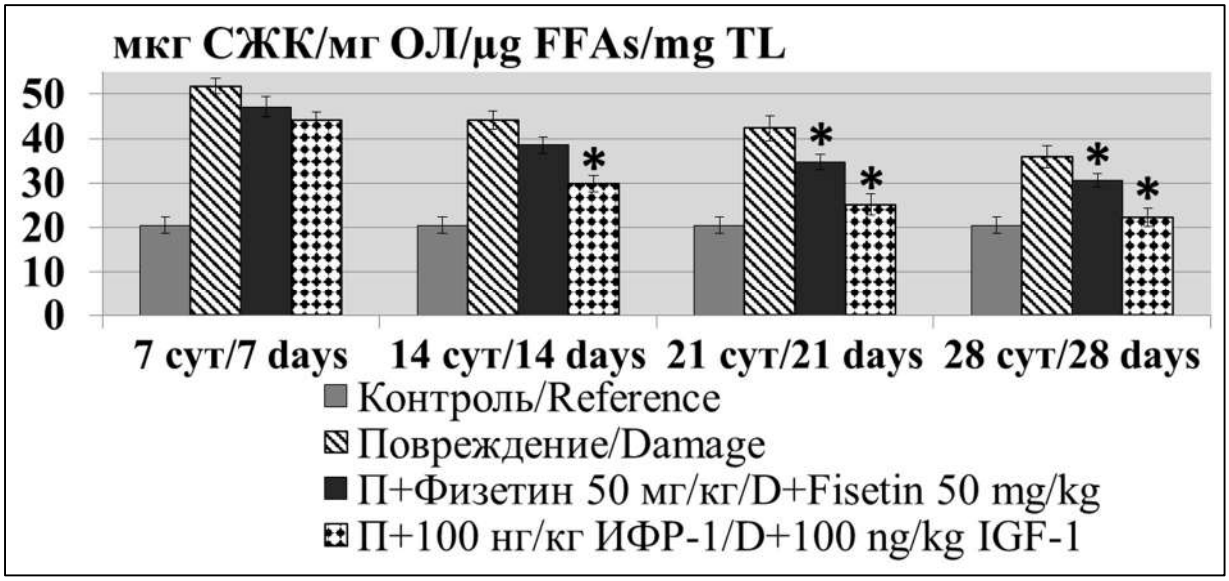

Fig. 3 - Effect of fisetin and IGF-1 on the change in the SFA content in the proximal and distal parts of the rat sciatic nerve after its injury (all error bars indicate the standard error of the mean value (n = 10), *- reliability of the difference in relation to the injury, $p<0.05$)

Similar dynamics can be traced in the distal part of the nerve conductor, but these changes are more pronounced due to deep degeneration processes along its entire length.

When fisetin is administered at a concentration of 50 mg/kg in the proximal nerve section, a decrease in the level of SFAs is observed: the amount of free fatty acids decreases by 18.0 % and 14.8 % compared to the damage by the 21st and 28th days, respectively (Fig. 3). In the same variant

of the experiment against the background of IGF-1 use at a concentration of 100 ng/kg, there is a decrease in the level of FFAs. By the 14th and 21st days of the experiment the concentration of this fraction decreases by 32.1 % and 40.4 %, respectively, in comparison with the damage, and with increasing the duration of the experiment up to 28 days the level of SFA decreases 1.6 times in comparison with the series of experiments with transection. It should be noted that in the distal segment of the nerve conductor there is a slight decrease in the level of SFA by the 21st day of observation against the background of the action of both drugs in their maximum concentrations, in other variants of the experiment no significant changes are observed.

The physicochemical state of the lipid bilayer plays an important role in the regulation of ion permeability, which is known to determine nervous and muscular excitability. Disturbance of lipid bilayer composition and structural rearrangements resulting from phospholipase activation are accompanied by changes in its physicochemical state. As a result of the trauma, the nerve conductivity is markedly reduced in the proximal part of the nerve and completely lost in the distal part of the nerve conductor. Proceeding from this, at the next stage of the study we evaluated the physicochemical state of the nerve conductor membrane in its proximal segment using Raman spectroscopy. It was found that the ratio of intensities of peaks I_{1122}/I_{1076} of the Raman spectrum reflects the ordering of fatty acid chains forming the hydrophobic layer in the membrane (Fig. 4).

Figure 4. Raman spectra of rat sciatic nerve fibre in the control

It is reported in the literature that when this index decreases, orderliness decreases, which is accompanied by a decrease in membrane viscosity [24]. The results of the experiments showed that the minimum value of this index is observed by the 7th day of the experiment: the degree of orderliness of nerve fibre membranes decreases by 86.8% relative to the control. In a series of experiments with intramuscular injection of fisetin in concentration of 50 mg/kg the value of the investigated index increases on the average by 22,7 and 25,2 % in comparison with the damage by

the 21st and 28th days respectively. In a series of experiments with IGF-1 administration at the initial terms after transection the ratio I_{1122}/I_{1076} practically does not change. By the 14th day of observation, the value of the ratio increases by 33.9 % compared to the injury. 28 days after injury, the ratio of I_{1122}/I_{1076} peaks increases by 28.8 % relative to the series of experiments with injury (Fig. 5).

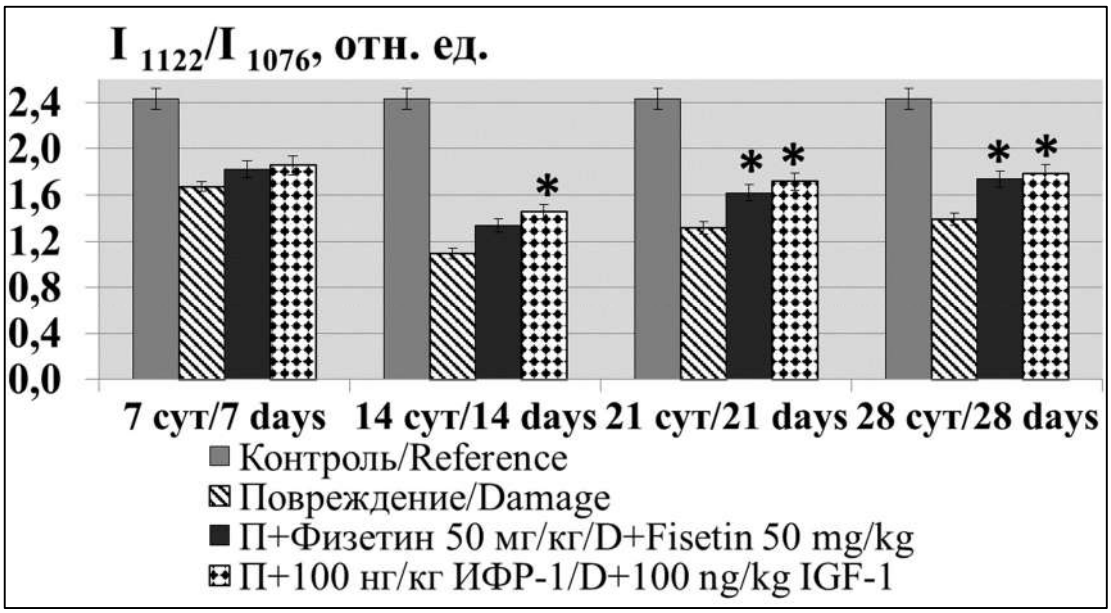

Figure 5. Effect of fisetin and IGF-1 on the change in the intensity ratio of I_{1122}/I_{1076} bands of the CR spectrum of the proximal part of the sciatic nerve after its injury: relative units. - relative units (all error bars indicate the standard error of the mean value (n = 10), in % of control; *- reliability of the difference in relation to control, $p<0,05$)

The physicochemical state of the lipid bilayer can also be judged by the ratio of the intensity of I_{2940}/I_{2853} bands, which characterises the ratio of the protein component to the lipid component (Fig. 4) [25, 26]. The experiment showed that transection of the sciatic nerve leads to a 3.7-fold decrease in the ratio $I_{2940}/I_{(2853)}$ relative to the control by the 7th day of the experiment as a result of the development of Waller degeneration processes, which is accompanied by proteolytic breakdown and destruction of the lipid fraction [17]. With the increase of postoperative terms up to 28 days, the value of this index increases, but still remains below the control values on average by 1.7 times. In a series of experiments with fisetin administration, a reliable increase in this index is observed when the drug is used at a concentration of 50 mg/kg for longer periods after transection: the intensity ratio of I_{2940}/I_{2853} peaks increases on average by 58.3 and 61.0 % compared to the damage by the 21st and 28th days of observation, respectively. In the series of experiments with IGF-1, a less pronounced increase in this index is observed compared to the use of fisetin: by the 21st day of observation, its value increases by 41.2%, and by the 28th day - by 38.5% compared to the damage (Fig. 6).

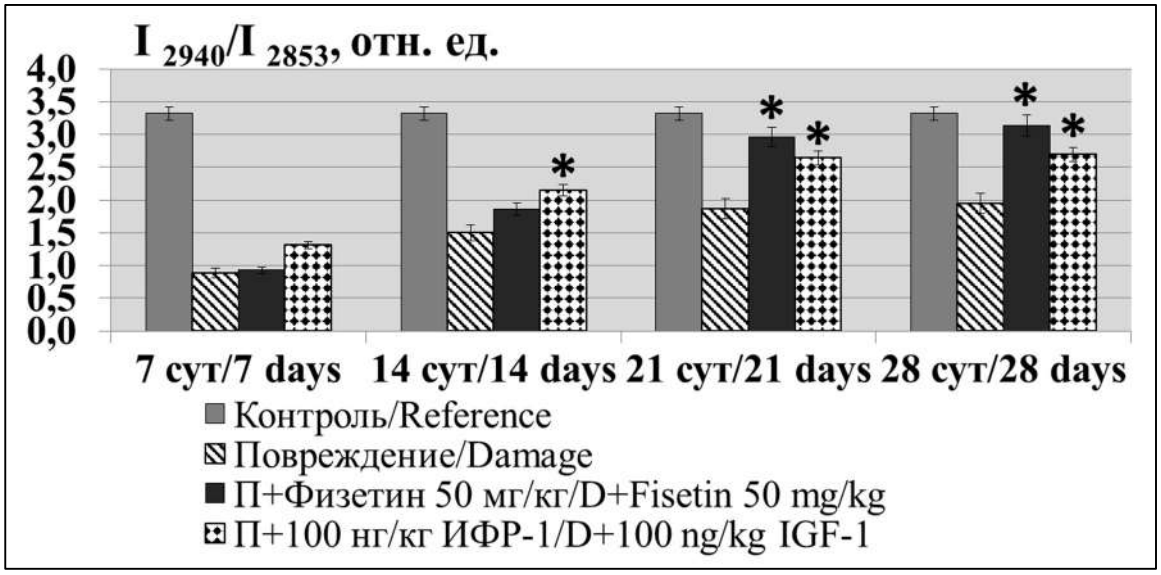

Fig. 6 - Effect of fisetin and IGF-1 on the change in the intensity ratio of $I_{2940}/I_{(2853)}$bands of the CR spectrum of the proximal part of the sciatic nerve after its injury: relative units. - relative units (all error bars indicate the standard error of the mean value (n = 10), in % of control; *- reliability of the difference in relation to control, $p<0,05$)

In addition to changes in the physicochemical state of the lipid bilayer of the nerve fibre, the most important evidence of the regeneration processes of damaged somatic nerves is the restoration of their ability to conduct the action potential. It was shown that as a result of transection, the amplitude of excitation conduction along the nerve is significantly reduced in its proximal section and completely lost in the distal section. With increasing the time of the damaging effect up to 28 days, the restoration of the ability to conduct the action potential with a small amplitude is noted only in the proximal segment of the nerve, which is apparently explained by the preservation of central innervation and partial restoration of neuromuscular transmission. In the variant of the experiment with intramuscular injection of fisetin in concentration of 50 mg/kg, an increase in conduction in the proximal segment of the nerve by 28 days of the experiment by an average of 40% in comparison with the damage was observed. The use of insulin-like growth factor-1 at a concentration of 100 ng/kg in the same variant of the experiment is accompanied by an average 1.6-fold increase in the amplitude of the action potential relative to the series of experiments with transection (Fig. 7).

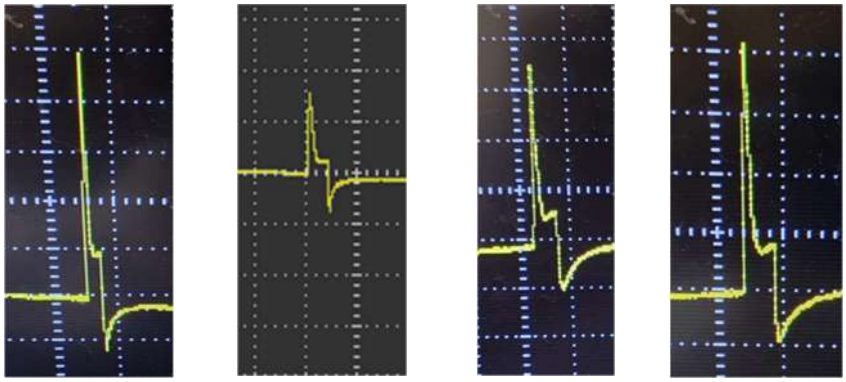

Fig. 7 - Action potential of the rat sciatic nerve. Potential scale: 20 mV per cell; time scale: 1.0 ms per cell: A) control nerve; B) proximal nerve 28 days after injury; C) proximal nerve 28 days after injury and administration of 50 mg/kg fisetin; D) proximal nerve 28 days after injury and administration of 100 ng/kg IGF-1.

Conclusion

From the results obtained we can state that the administration of fisetin and insulin-like growth factor-1 enhances regenerative processes in the injured nerve conduit, as evidenced by the restoration of the ability to conduct the action potential and an increase in the ordering of the lipid bilayer of the nerve fibre after injury in the proximal segment of the nerve under the action of the studied drugs. Nevertheless, the mechanisms of their action remain different. It is known from the literature that the effect of IGF-1 on cells is determined by its binding to IGF-receptor type 1 (IGF-R1), which is expressed in all cell types except hepatocytes and T-lymphocytes and is an important element of normal development of the organism. Recent studies have demonstrated that mouse embryos deprived of IGF-R1 have developmental defects in lung, skin, bone, and neurological abnormalities [27]. It was shown that intramuscular administration of insulin-like growth factor-1 promotes intensification of phosphoinositide metabolism, accumulation of DAG and reduction of FGF levels. Our results are consistent with the literature data indicating the ability of IGF-1 to trigger several signalling pathways associated with the formation of lipid metabolites. One of them is mediated by activation of phosphoinositide-specific phospholipase C, resulting in a decrease in the quantitative content of phosphatidylinositol-4,5-diphosphate (DFI). The breakdown of DFI is accompanied by the formation of diacylglycerol and inositol triphosphate, which activates Ca^{2+}-channels of intracellular depots through which Ca^{2+} is released into the cytoplasm. Diacylglycerol remains in the plasma membrane and Ca^{2+}-dependently activates protein kinases of the PKC and PKD families, as well as a number of ion channels. This signalling pathway is known to regulate such important physiological functions as cell growth, proliferation and differentiation [28].

When fisetin was administered, insignificant changes in the content of FI and DAG were observed throughout the entire period of the experiment in both proximal and distal sections of the nerve conductor. Besides, the data of CR-spectroscopy indicate the activation of protein synthesis under the action of fisetin, which may indicate the absence of the effect of fisetin mediated through phosphoinositide-specific phospholipase C on the change in the level of phosphoinositide cycle components.

Thus, under the action of insulin-like growth factor-1 there is an intensification of the exchange of phosphoinositide cycle components, indicating a phospholipase C - mediated mechanism of IGF-1 action in injured somatic nerves, leading further to the formation of components of the phosphatidylinositol-3-kinase signalling pathway involved in the stimulation of the expression of various transcription factors necessary for axonal regeneration and restoration of the functioning of injured nerve conductors [29]. In contrast to IGF-1, the absence of intensification of phosphoinositide metabolism and increased synthesis of protein fraction under fisetin action indicates acceleration of regeneration processes in the injured nerve conductor, most likely caused by activation of the mitogen-activated protein kinase signalling pathway [30], which is accompanied by expression of genes associated with remyelination and restoration of the physicochemical state of the nerve fibre membrane due to protein synthesis.

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