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INFLUENCE OF POTASSIUM HYALURONATE ON THE CHANGE OF PHOSPHOLIPASE ACTIVITY AND STATE OF THE MEMBRANES OF DAMAGED SOMATIC NERVES OF RATS

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ABSTRACT

It has been established that nerve injury is accompanied by an increase in the content of lysophospholipids and an increased activity both Ca^{2+} - dependent and Ca^{2+} - independent phospholipase A_2. The introduction of the drug enhances the regenerative processes in damaged nerve that is manifested in the restoration of orderliness of the membranes of nerve fibers, as well as in the reduction of lysophospholipids and activity of Ca^{2+} - dependent phospholipase A_2. However, using the potassium hyaluronate does not lead to changes in the activity of Ca^{2+} - independent phospholipase A_2 that likely indicates that Ca^{2+} - independent phospholipase A_2 is not involved in the mechanism of nerve regeneration. Thus, it can be assumed that the acceleration of regeneration processes in damaged nerve conductor under the action of potassium hyaluronate is likely mediated by the functioning of Ca^{2+} - dependent phospholipase A_2.

KEYWORDS: Phospholipase A_2, potassium hyaluronate, lysophospholipids, nerve regeneration, Raman scattering spectroscopy.

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INTRODUCTION

The problem of recovering the function of damaged peripheral nerves is still relevant due to insufficient efficiency of different approaches and therapies[1,2]. Taking into account the importance of this problem, various ways to optimize the axonal regeneration, have been actively searched for. A very promising trend for posttraumatic nerve regeneration is the use of biologically active substances, particularly hyaluronic acid[3,4]. There are more and more studies on use of the drugs based on hyaluronic acid[5,6]. It is reported that the high molecular weight form of hyaluronic acid provides the proliferation and self-renewal of neural stem cells[7]. It is known that hyaluronic acid plays an important role in the development of central nervous system due to binding to the CD44 and RHAMN specific receptors of different cell populations[8]. The therapy of damaged nervous system with using hyaluronic acid supports and enhances the neurite outgrowth, generally contributing to a successful recovery of cell membranes' properties[9]. However, the mechanism of action of hyaluronic acid in damaged somatic nerves is still unknown. Taking into consideration the limited data on the participation of hyaluronic acid in reducing the hydrolysis of phospholipids due to decreasing the activity of phospholipase A_2 in acute lung inflammation and osteoarthritis[10], we can assume the existence of phospholipase A_2 – mediated mechanism of action of hyaluronic acid. In this regard, the aim of our work was the study of change in activity of phospholipase A_2 and the status of rats' somatic nerves under the damage and action of potassium hyaluronate – the drug based on hyaluronic acid.

MATERIALS AND METHODS

The experiments were carried out on outbred rats weighing 200-250 g, which were housed on the standard food ration. The use of animals must be in accordance with the ILARGuide for Care and Use of Laboratory Animals[11]. The mechanical injury was implemented using the ligature at the level of midthigh. In animals of the first group anesthetized with diethyl ether, the sciatic nerve was exposed and ligated. In animals of the second group, the solution of potassium hyaluronate was applied to the damaged sciatic nerve (Hyaluronic acid potassium salt from human umbilical cord, Sigma) at concentrations of 2 mg/kg, 17 mg/kg and 30 mg/kg. The sciatic nerves were removed after 12 hours, 1 day, 3 days, 7 days and 30 days. Intact animals were used for reference. The activity of phospholipase A_2 (PL A_2) was determined by accumulation of free fatty acids, composition of which was analyzed using a Shimadzu GS 2010 gas chromatograph (Japan). Reaction medium for determining the activity of Ca^{2+} - dependent phospholipase A_2 contained 10 mM Tris, 0.05 M NaCl, 5 – 20 мМ $CaCl_2$, 0.5% solution of detergent Triton X-100, pH 8. Reaction medium for determining the activity of Ca^{2+} - independent phospholipase A_2 contained 10 mM Tris, 0.05 M NaCl, 1 mM EGTA, 0.5% solution of detergent Triton X-100, pH 8. Phosphatidylcholine extracted from egg yolk and purified by chromatographic procedure using the chloroform/methanol/acetone/ glacial acetic acid/water (40:13:15:12:8, by volume) system was used as a substrate. The specific activity was expressed in micrograms of fatty acids formed during 1 hour per mg of protein. The content of protein was determined according to the method of Lowry[12]. Lipids were removed from the nervous tissue according to the method of Bligh-Dyer[13]. Phospholipids were separated using the method of one-dimensional chromatography on silica gel in the chloroform/methanol/ammonia/water (65:30:4:2) system of solvents[14]. Phospholipids were quantitatively determined using densitometric method in the CAMAG TLC Scanner 4 automated complex (Switzerland). Change in the status of the membranes was detected by a Renishaw Raman spectrometer (UK). One-way analysis of variance was used, followed by the Student's t-test. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

The study has shown that the average activity of PL A_2 is 18 μg/fatty acids/mg protein/hour. However, in as little as 12 h after the injury, the activity of PL A_2 exceeds the reference

value by 6.8 times in the average. An increase in the duration of damaging interference is accompanied by increasing of enzyme activity. So, the maximum enzyme activity occurred by 7 days and was 482 µg/fatty acids/mg protein/hour. With increasing the post-surgical period up to 30 days, the activity of PL A_2 is reduced, but still exceeds the reference value by 110% (Fig 1). It has been established that PL A_2 catalyzes hydrolysis of phospholipids with accumulation of lysophospholipids (LPL) and free fatty acids (FFA)[15,16], which are one of the features of deep degenerative processes[15,17,18,19]. The research has shown that the maximum accumulation of lysophosphatidylcholine (LPC) is found on the 7th day and is 27.3 µg P_{LPC}/mg P_{PL} in the average. By the 30th day the LPC content in damaged nerve was slightly decreased and was still 2.4 times higher than the reference value. In the fraction of lysophosphatidylethanolamine (LPEA) the similar dynamics are observed. In a series of experiments with introducing potassium hyaluronate, the phospholipase activity also increased, but to a lesser degree, as compared to the injured nerve without the influence of the drug. It has been established that potassium hyaluronate (PH) in low concentrations has virtually no effect on changing the phospholipase activity. Veracious reduction of phospholipase activity is observed in case of using the drug at a concentration of 30 mg/kg. Moreover, the most pronounced effect of PH is observed long time after the nerve damage. So, by the 7th day of the observation, the PL A_2 activity in a series with PH is reduced as compared to the ligature by 58.3% in the average. It should be noted that with increasing the post-surgical period to 30 days the enzyme activity in a variant with PH decreases by 1.6 times as compared to the injury and does not significantly differ from the reference (Fig 1).

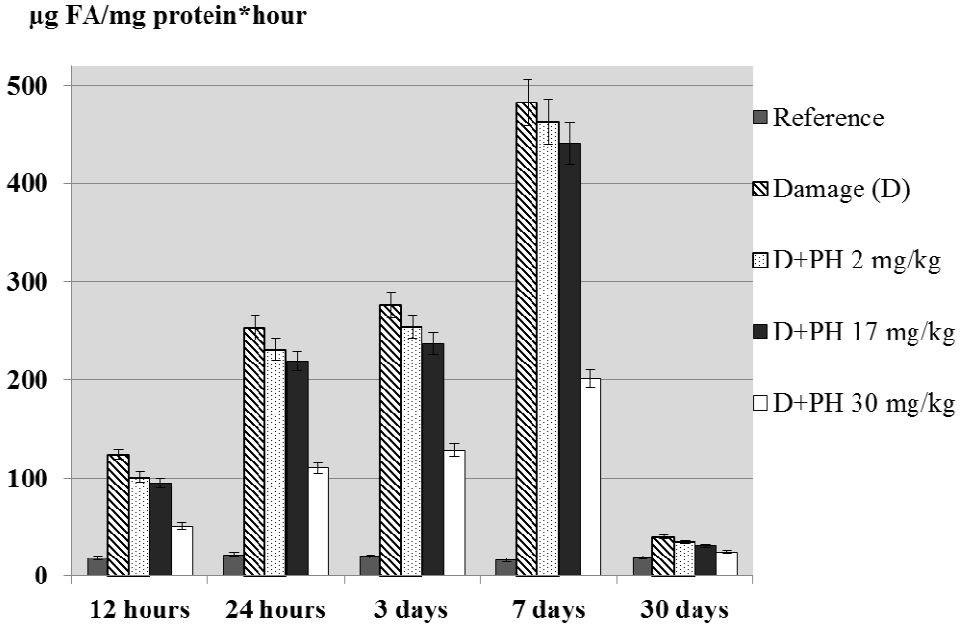

Figure 1
Change in the activity of phospholipase A_2 during incubation of the sciatic nerve in the medium with Ca^{2+}: D+PH 2 mg/kg - damage+potassium hyaluronate in concentration 2 mg/kg, FA – fatty acids

Because of the direct relationship between phospholipase activity and LPL content we investigated the influence of the drug on the change of LPC and LPEA level in the next series of experiment. It has been established that PH in the maximum concentration has the most pronounced effect after 3 and 7 days and contributes to reduction of LPC level by 23.2 and 52.1% respectively as compared to the injury. With increasing the post-surgical period up to 30 days the content of LPC in a variant with PH significantly decreases and virtually does not differ from the reference values. The similar dynamics were observed in case of influence of the drug on the LPEA content (Fig 2).

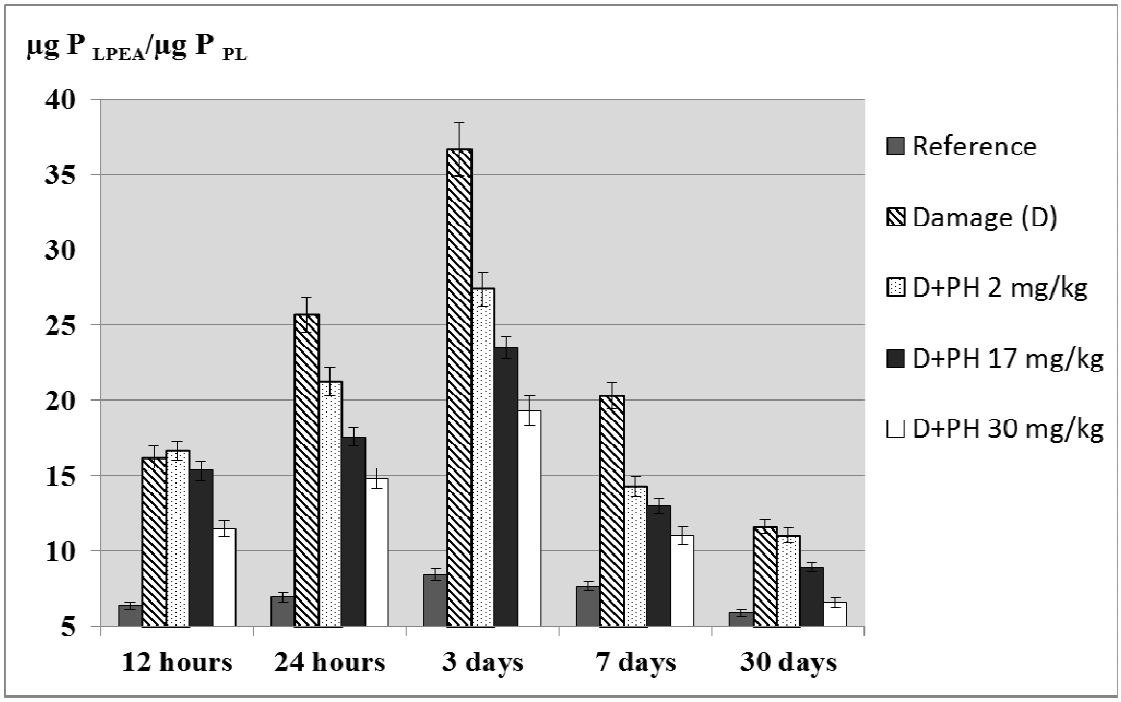

Figure 2

The effect of potassium hyaluronate on changing the content of LPEA in the sciatic nerve after injury: µg P $_{LPEA}$/µg P $_{PL}$ - µg of inorganic phosphorus of lysophosphatidylethanolamine/µg of inorganic phosphorus of phospholipids

At the next stage we have performed a series of experiments in a medium with ethylene glycol tetraacetic acid (EGTA) to bind intracellular calcium and determine the activity of Ca^{2+} - independent phospholipase A_2 in damaged nerve conductor. It has been established that in medium with EGTA the phospholipase A_2 activity exceeds the reference value by 1.8 times 12 hours after trauma. 3 days after injury the activity increases by 3 times, however the maximum phospholipase activity in damaged nerve is found by 7 days of the observation and was 105 µg/fatty acids/mg protein/hour. Thus, the ligature of the nerve is accompanied by a rapid increase of PL A_2 activity and with increasing post-surgical period to 30 days the phospholipase activity decreases but still exceeds the reference value. In a group of animals with introducing potassium hyaluronate no veracious change of phospholipase activity in a medium with EGTA is observed (Fig 3).

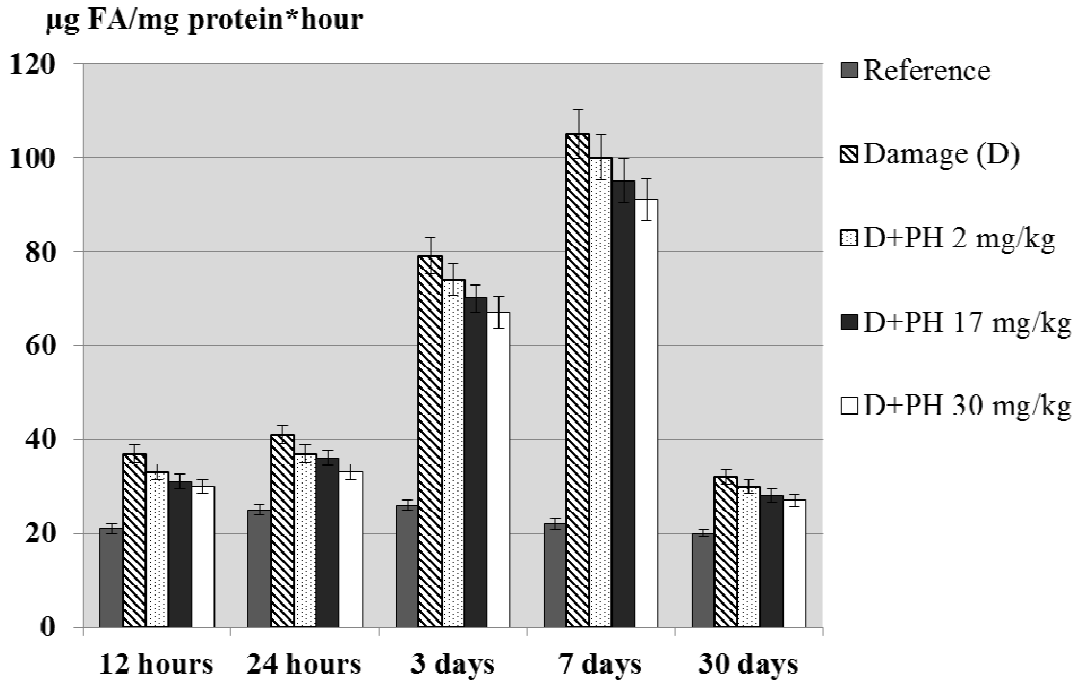

Figure 3
Change in the activity of phospholipase A_2 during incubation of the sciatic nerve in the medium with EGTA

At the next stage of the study using the Raman scattering spectroscopy method, we evaluated the ratio of intensities of peaks I_{1122}/I_{1076} (Fig 4) that reflects the extent of orderliness of membranes of nerve fiber.

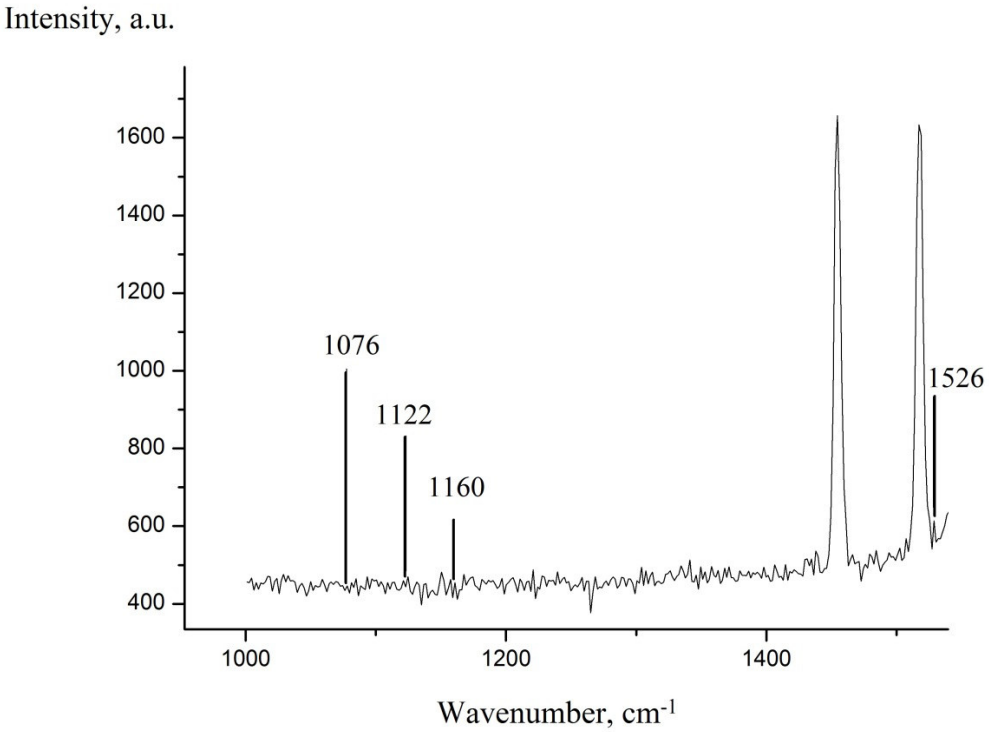

Figure 4
The Raman spectrum of the sciatic nerve fibers in the reference

When this indicator decreases the orderliness also decreases that is accompanied by reduction of membrane viscosity[20]. The experimental results have shown that the maximum value of this indicator is found by the 7th day of the experiment: the extent of orderliness of membranes of nerve fiber is decreased by 57.1% compared to the reference. In a series of experiments with PH the value of the indicator increases to the end of the experiment (Fig 5).

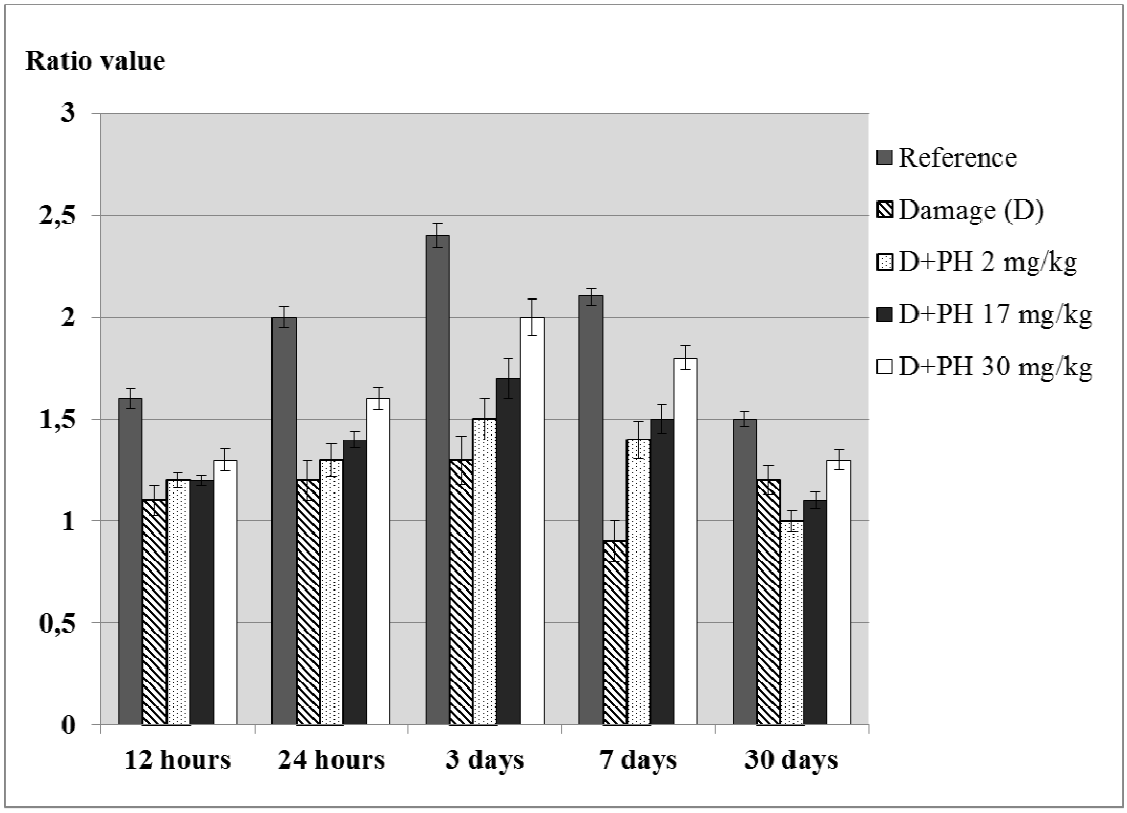

Figure 5
Histogram comparing I_{1122}/I_{1076} ratio of intensity between different groups

The orderliness of lipid bilayer can also be judged by the indicator of intensities of the I_{1526}/I_{1160} (Fig 4) bands, which characterizes the absorption spectrum of carotenoids[21]. It is known that this indicator directly depends on the extent of orderliness of fatty acids' "tails" of membrane lipids. The experiment has shown that the ratio of intensities of peaks I_{1526}/I_{1160} reduces up to the 7th day of observation by 62.5% as compared to the reference group. However, increasing the duration of damaging effect to 30 days leads to an increase of orderliness of fatty acids in the membrane by 1.5 times compared to the injury. In a series of experiments with introducing of PH a veracious increase of the indicator is observed in case of using the drug at a concentration of 30 mg/kg (Fig 6).

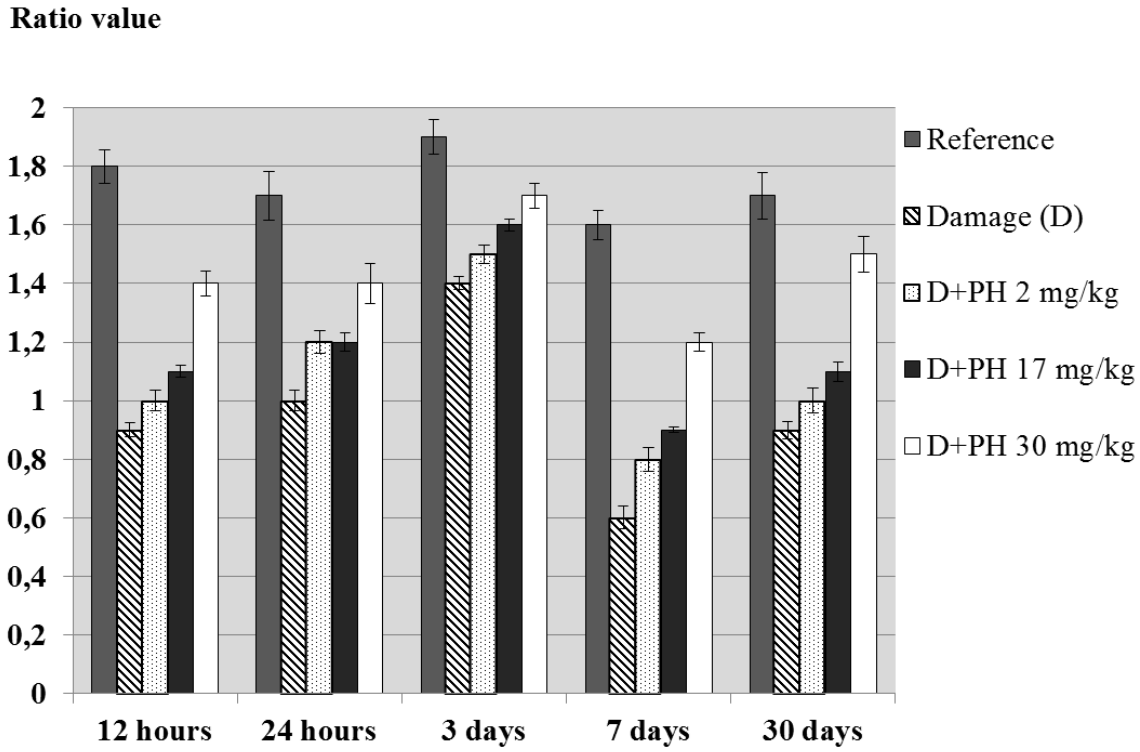

Figure 6
Histogram comparing I_{1526}/I_{1160} ratio of intensity between different groups

DISCUSSION

Taking into account the literature data[10, 22, 23] and the results of our own study we can assume that the expression of membrane protective properties of potassium hyaluronate is implemented through the regulation of the activity of membrane-bound phospholipase A_2. There are numerous studies that indicate the possible involvement of PL A_2 in the degenerative processes occurring in the nerve conductor[24]. So, Ca^{2+} - dependent phospholipase A_2 is involved in the activation of engulfment of myelin residues by macrophages, which is a necessity condition for the processes of axonal regeneration[23]. The results have shown that the injury of sciatic nerve is accompanied by an increase of phospholipase activity. In this regard, the maximum enzyme activity was detected by 7th day of observation that correlates with the literature data, which indicates the end of axonal degeneration by the 5-7th day after nerve injury[15]. By the 30th day of the experiment the enzyme activity is a little reduced but still significantly exceeds the reference values. Apparently, the reduction of PL A_2 activity is due to the reparative processes in myelin sheathes of nerve fibers, which may last up to 50 days of observation[6]. According to the literature date the enzymes from the family of intracellular phospholipase A_2, namely Ca^{2+} - dependent and Ca^{2+} - independent can play an important role in wallerian degeneration at early stages of damage of myelin sheathe[23]. In this regard, we have performed a series of experiments for determining the activity of Ca^{2+} - independent phospholipase A_2. Study has shown that during incubation of sciatic nerve of rat in the medium with EGTA, the enzyme activity reduces in all variants of experiments with the injury as compared to a similar series in the medium with Ca^{2+}. Nevertheless, the manifestation of enzyme activity in the absence of calcium ions allows suggesting that both forms of PL A_2 are included in the sciatic nerve and took part in the degenerative processes. It is known that hyaluronic acid plays a crucial role in protecting phospholipids of synovial fluid from the lysis carried out by phospholipase A_2. The mechanism of action of hyaluronic acid in synovial fluid described up to date is mediated by receptors and includes the inhibition of inflammatory mediators and phagocytic cell function[25]. However, the

mechanism of action of potassium hyaluronate in damaged peripheral nerve system is still poorly understood. We hypothesized that PH accelerates the regeneration processes in the damaged nerve conductor and one of the mechanisms of this process is realized through PL A_2. In order to check this assumption, we carried out a series of experiments for determining the phospholipase activity under the action of the drug. It has been established that PH at a concentration of 30 mg/kg caused a significant decrease in enzyme activity compared to the injured nerve without drug application (Fig 1). With that, the most pronounced effect of PH is observed at early stages of myelin degradation, and an increase of post-surgical period up to 30 days is accompanied by significantly reduction of enzyme activity, its value is closed to the reference. The obtained data are also compatible with the data, which indicate the consistent necessity of PL A_2 activity for outgrowth of growth cones of axons in the peripheral nerves of rats[18]. It is known that activity of the majority of membrane enzymes, including enzymes from a family of phospholipase A_2 is determined by viscosity of lipid bilayer[15,26]. According to the data of Raman spectroscopy an increase of viscosity and orderliness of the membranes of nerve fibers under the drug is observed that indicates the manifestation of regenerative effect of PH. Nevertheless, it is still unclear whether the development of regenerative processes under the action of PH is due to activation of Ca^{2+} - dependent phospholipase A_2 or Ca^{2+} - independent phospholipase A_2 that is also involved in this mechanism. Based on this we carried out a series of experiments for determining the Ca^{2+} - independent phospholipase A_2 during the nerve incubation in the medium with EGTA. The experiment has shown that in case of nerve injury an increase in activity of Ca^{2+} - independent phospholipase A_2 is observed. However, in a variant with PH no significant differences of enzyme activity compared to the injury have been found (Fig 3). No changes in activity of Ca^{2+} - independent phospholipase A_2 in case of introducing the drug likely indicate that Ca^{2+} - independent phospholipase A_2 is not involved in the mechanism of nerve regeneration.

CONCLUSION

As it is seen from results, the introduction of potassium hialuronate enhances the recovering processes in damaged nerve that is accompanied by a decrease in activity of Ca^{2+} - dependent phospholipase A_2 and a less pronounced accumulation of lysophospholipids. In addition, Raman spectroscopy data indicate an increase in the viscosity of the lipid bilayer of the injured nerve membranes in case of introducing of the drug. Taking into account the results of our research and the literature date we may presume that the acceleration of regeneration processes in damaged nerve conductor under the action of potassium hyaluronate is likely mediated by the functioning of Ca^{2+} - dependent phospholipase A_2.

CONFLICT OF INTEREST

Conflict of interest declared none.

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