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^{31}P NMR Manifestation of Metabolic Changes in Blood of Spinal Cord Injured Rats

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Abstract The ^{31}P nuclear magnetic resonance spectroscopy technique was applied to study the blood of rats with a 3-day spinal cord injury and control rats. Phosphorus-containing blood metabolites: 2,3-diphosphoglycerates, inorganic phosphates, phospholipids, ATP and adenosine monophosphates were detected and quantitatively evaluated. It was found that the amount of 2,3-diphosphoglycerates, inorganic phosphates, phospholipids and adenosine monophosphates increase, and pH of the blood decreases after spinal cord injury. The results demonstrate increased hypoxia in injured rats.

1 Introduction

The traumatic injury of the spinal cord is one of the most acute problems of neuroscience and medicine. Despite this for today, there are no effective methods for treating spinal cord injury (SCI) and one of the reasons for this is a multifactor nature of molecular mechanisms in SCI. Morphological investigations of injured spinal cord indicate that the destruction of tissue is not limited to the area of exposure, but it continues in time, capturing the primary intact parts of the brain, and forming a lesion larger than the initial trauma area. The search for new approaches to the treatment of the SCI encourages researchers to deeper and more detailed study of the mechanisms of pathogenesis of traumatic spinal cord disease. So, an application of novel methods for obtaining new molecular information which is inaccessible in

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using the traditional biochemical and clinical methods of investigation and diagnosis is actual.

One of the sources of new metabolic experimental data about SCI may become the ^{31}P nuclear magnetic resonance (NMR) spectroscopy of blood, as it enables to obtain important information about metabolic shifts in the development of various pathological conditions by noninvasive method. ^{31}P NMR spectroscopy allows to characterize organic and inorganic phosphorus compounds in blood, such as: 2,3-diphosphoglycerates, inorganic phosphates, phospholipids, ATP and some others. Phosphorus compounds participate in regulation of the acid–base state, in metabolic processes of substances (proteins, fats, carbohydrates); they are the most important accumulators and carriers of energy. Thereby the change of their profile can reflect the variation in general condition of the organism. The method of ^{31}P NMR spectroscopy allows to study the problems associated with the changes in hypoxia, energetic metabolism, and to assess the pH level in cells nondestructively.

The study was done on the samples of whole blood, as the most available diagnostic material to estimate the state of the organism in conditions of SCI. NMR data on phosphorus metabolites in blood allow to monitor the general condition of the organism in dynamics.

A significant part of phosphorus compounds is present in erythrocytes. Due to erythrocytes are the most abundant cells in blood, the main physical–chemical properties of blood are determined mainly by these cells. And since the erythrocyte, in particular its membrane, is a kind of "mirror" reflecting the state of the cells of other tissues and body systems, the study of blood phosphorus compounds by NMR is interesting for assessing the state of the organism after SCI. The quantitative evaluation of phosphorus-containing compounds in blood after SCI, in particular 2,3-diphosphoglycerates in erythrocytes, can provide a valuable information about hypoxia, which is important for understanding the pathophysiology of spinal cord injury.

Previously, ^{31}P NMR spectroscopy was used in studies of erythrocytes, blood plasma and whole blood [1–7]. NMR blood tests of patients with various pathologies were conducted [3, 5, 8–10] and the blood of laboratory animals, was also studied by this technique [4]. It was shown that ^{31} P NMR spectroscopy makes possible to quantify the change in phosphorus-containing compounds in the blood and may have prognostic importance in various diseases.

^{31}P NMR spectroscopy was used to study changes in phosphorus metabolism directly in spinal cord in vivo after experimental SCI in animals [11–13]. There was a decline in intracellular pH, a loss of high-energy phosphates, and emergence of a resonance from myelin phospholipids in first hours after injury. It was shown, that it is possible to predict irreversible tissue damage, basing on early changes in metabolism detected by NMR spectroscopy.

The aim of this study was to identify phosphorus metabolites in blood by ^{31}P NMR spectroscopy, to evaluate the intracellular pH under physiological and SCI conditions. It could be of interest to establish which characteristics of phosphorus metabolism change after SCI, and how they characterize the molecular biochemical processes. The obtained information could be used to monitor cellular phosphorus metabolism, as well as to assess the general condition of the organism after SCI.

The use of NMR technique to study blood metabolites in SCI also makes possible to observe the process in dynamics and to correct the tactics of treatment.

2 Experimental

2.1 Samples

In this investigation we used blood samples from rats of two groups—control rats ($n = 7$) and rats with the experimental 3-day SCI model ($n = 7$). The period of 3 days after SCI corresponds to the end of the acute phase of the traumatic process. The SCI was modeled by the Allen's method [14]. Venous blood sampling was performed from the right ventricle of the heart in 3 days after the injury. The blood was taken into syringes washed out with 5% sodium citrate solution to prevent blood clotting. All the manipulations were carried out in compliance with bioethical norms in rats. The animals were anesthetized with combined intramuscular anesthesia using "Zoletil 50" 1 mg/kg and "Xilavet" 0.10 ml/kg. 0.7–0.8 ml of blood was placed into standard vacuum ampoules, treated with blood anticoagulant—sodium salt of ethylenediamine-tetra-acetic acid (EDTA), and was delivered to the NMR laboratory in such form. No later than in 30 min after blood sampling, the blood was placed in special 5-mm glass tubes for NMR measurements. The amount of blood in the sample was always the same: 60 mm column in standard NMR tube. NMR spectra of the whole blood were measured under the same conditions in an hour after blood sampling.

2.2 NMR Measurements

All NMR experiments were performed at the temperature 20 °C on an "Avance 400" NMR spectrometer manufactured by Bruker (Germany). The carrier frequencies for the ^{1}H nuclei were 400 MHz and for ^{31}P nuclei were 162 MHz. The durations of 90° pulses for the ^{1}H and ^{31}P nuclei were 11 and 14 μs, respectively. The width of ^{1}H spectra was 5.3 kHz and of ^{31}P spectra 15 kHz. The number of points used during the registration of FID was 8192 for both types of spectra. The number of scans for the ^{1}H spectra was 16, and for the ^{31}P spectra it was 1024. The cycle delay was 5 s for ^{1}H spectra and 3 s for ^{31}P spectra. As the chemical shift reference for ^{1}H nuclei the signal of residual protons in D_2O was used, whereas for ^{31}P nuclei the signal of 85% H_3PO_4 was used. Data acquisition and processing of spectra were carried out by the Bruker XWIN-NMR 3.5 software.

2.3 Results

The whole blood samples of control rats ($n = 7$) and rats in 3 days after SCI ($n = 7$) were studied by NMR spectroscopy. In the ^{31}P NMR spectra of rat blood four to six lines were observed. It is known that the main signals in the ^{31}P NMR spectrum of

whole blood are those from 2,3-diphosphoglycerates, inorganic phosphates, adenosine triphosphate (ATP) molecules.

In this study, in the typical NMR spectra of blood in the region of positive chemical shifts, four main intensive lines 1, 2, 3 and 4 were recorded. In some cases (mainly in rats with a spinal cord injury), one or two weak lines (line 5, line 6) (Fig. 1) additionally appeared.

Intensive signals 1 and 2 are traditionally described as the signals due to 2,3-diphosphoglycerates (respectively 3- and 2-diphosphoglycerates) (2,3-DPG) [1–7]. 2,3-Diphosphoglycerates are the molecules formed under oxygen deficiency as an intermediate product of glycolysis. 2,3-DPG are able to bind to deoxyhemoglobin, decreasing the affinity of hemoglobin for oxygen and facilitating oxygen transfer to tissue cells. Therefore, the intensity of 2,3-DPG lines reflects the amount of deoxyhemoglobin in the blood and is an indicator of hypoxia. With increasing hypoxia in the organism, the intensity of these signals increases. Since 2,3-diphosphoglycerates are in erythrocytes, the lines of 2,3-DFG (lines 1, 2) are recorded only in erythrocyte samples and in whole blood [1–7], and they are not detected in plasma [2, 3].

During the storage of samples the intensity of the lines may vary, because some biochemical processes in blood samples are continued, but at a slower rate. Therefore, to select the correct regime of measurements, the dynamics of NMR blood spectrum alteration in 1, 2, 3 and 4 h and in a day after blood sampling was analyzed (Fig. 2). It was found that with the course of time the intensities of lines 1 and 2 demonstrate a slight decrease, therefore all the measurements were carried out under the same conditions no later than 1 h after blood sampling. The decrease in signal intensity during the first hour did not exceed 10%. The dynamics of the spectrum changes is shown in Fig. 2.

Signals 3 and 4 corresponded to inorganic phosphates (Pi). Signal 3 increased over the time, and signal 4 was more stable, it almost did not change the intensity

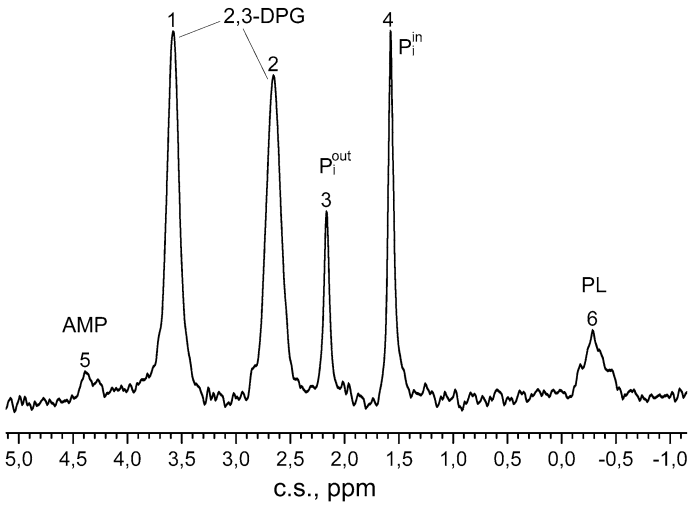

Fig. 1 Typical ^{31}P NMR spectrum of blood of an injured rat

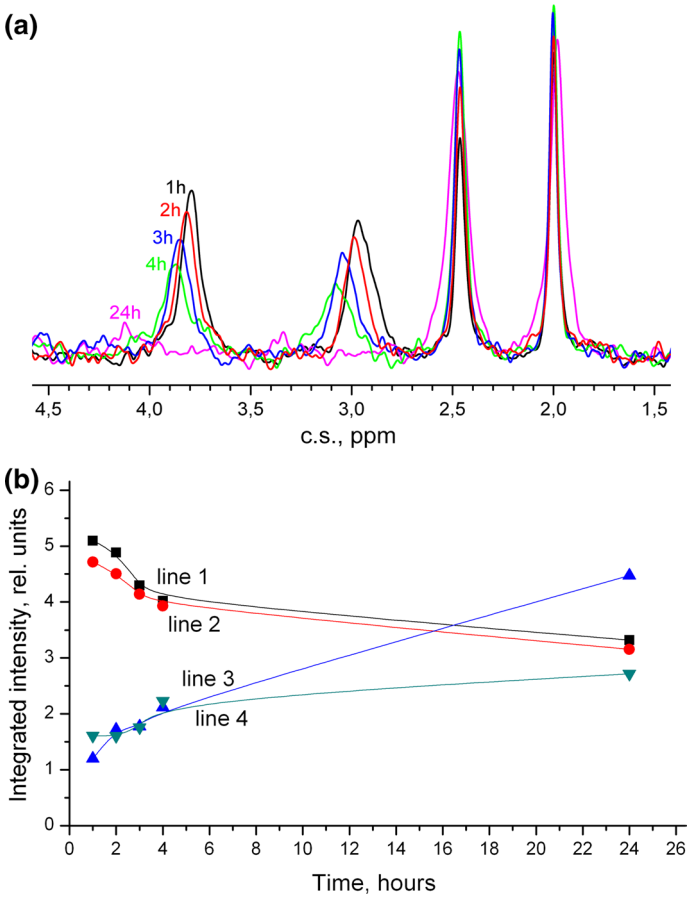

Fig. 2 Dynamics of the main ^{31}P NMR signals in blood of rat with SCI. Spectra were obtained in 1, 2, 3, 4 and 24 h after blood sampling, respectively. **a** Dynamics of ^{31}P NMR signals 1–4, **b** dynamics of their integrated intensities

during first 4 h. Hence it can be assumed that a more stable signal 4 corresponded to more stable chemical medium within the cells, whereas signal 3 corresponded to the extracellular environment, blood plasma; and signal 3 enhancement corresponded to the release of inorganic phosphate from organic phosphorus molecules in vitro.

In identification of the lines 3 and 4 it was also taken into account that the position of lines of inorganic phosphate depends on the medium pH [1], and the increase of a pH value is to result in the increase of the resonance line frequency. Since it is known that the average blood pH is about 7.4 and pH inside the body cells (including erythrocytes) is lower and varies between 7.0 and 7.2, then according to the dependence obtained in [1] the chemical shift of the inorganic phosphate in erythrocyte should be less than that located outside the cell (in the blood plasma). Consequently, line 3 corresponds to plasma and line 4 corresponds to erythrocytes. Thus,

lines 3 and 4 were unambiguously identified as lines of inorganic phosphates: line 3—outside cells, line 4—inside cells.

It should be noted that in studies of packed red blood cells [1, 2, 6–9, 15] or blood plasma [2, 3] only one line of inorganic phosphates was recorded. In our case as well as in whole blood studies [1, 5] and in ^{31}P NMR spectra in vivo in heart and blood [16] two different Pi signals were observed characterizing two pools of inorganic phosphates inside and outside the cells.

Line 5 (Fig. 1) similar to the data [7, 8, 17] corresponded to adenosine monophosphate (AMP) which is one of the decay products of ATP. This line was observed in the region of ~ 4.4 ppm, characterized by weak intensity and was more noticeable in the blood of rats with SCI.

Another weak line designated as line 6 (Fig. 1) was recorded in the region of negative chemical shifts at about − 0.3÷− 0.4 ppm. Line 6 corresponded to plasma phospholipids (PL) in accordance with [1, 2, 5, 15]. This line was mainly observed in blood NMR spectra in rats with SCI and it was not registered in NMR spectra in control blood samples (except one sample).

The typical ^{31}P NMR spectra of blood samples in control group and in group with SCI are shown in Fig. 3. The spectra of injured rats show the shift of lines 1–4 relative to control spectra. The values of chemical shifts and integrated intensities for all types of lines were estimated and their average values were calculated. They are given in Table 1.

Additionally to described lines 1–6 in negative chemical shifts region ^{31}P NMR spectra of the blood a few weak signals were also detected (Fig. 4) at about − 5, − 10, and – 19÷− 20 ppm. These lines corresponded to well known signals of adenosine triphosphates: γ-, α-, and β-phosphorus nuclei of ATP, respectively [2–5, 7–9]. However, these signals had a weak intensity that may be also related to either the small amount of studied sample or with a high viscosity. The low intensity of these signals did not allow us to detect any noticeable quantitative

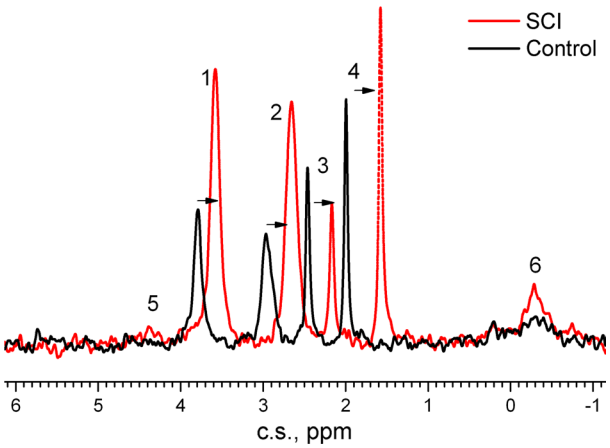

Fig. 3 Example of the ^{31}P NMR spectrum changes in blood of rat after SCI

Table 1 The characteristics of detected ^{31}P NMR signals in rat blood

^{31}P spectral line	1	2	3	4	5	6
The average value of the chemical shift, ppm						
Control ($n = 7$)	3.72	2.85	2.33	1.73	–	0.28
SCI ($n = 7$)	3.60	2.71	2.22	1.62	4.39	0.37
The average value of the integrated intensity, relative units						
Control ($n = 7$)	4.997	4.984	1.828	2.472	–	0.696
SCI ($n = 7$)	7.182	6.985	1.984	2.643	–	1.618

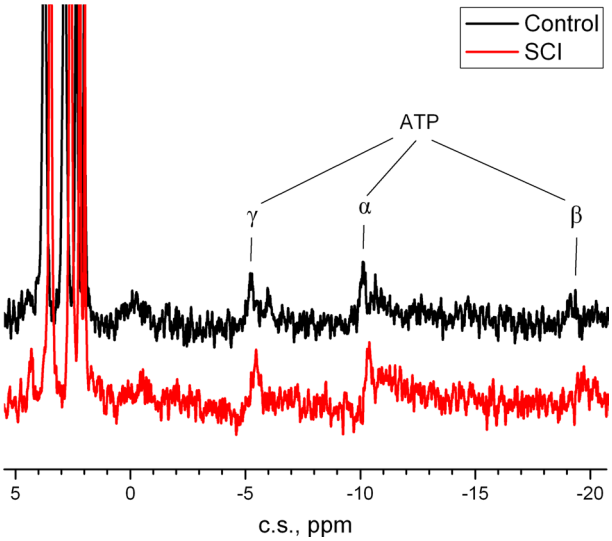

Fig. 4 ^{31}P NMR lines corresponding to the ATP molecule

change of the ATP in the blood of injured rats. Attempts to accumulate these signals during a longer time did not lead to the increase in their intensity; because it is known that the metabolic processes in the blood samples continue with time and even a slight shift of the lines due to these processes do not allow the signals to increase significantly.

Since the blood preservative sodium citrate could be present in trace concentrations in the blood samples, to avoid inaccuracies in chemical shifts estimation the effect of sodium citrate on the ^{31}P NMR blood spectrum was studied (Fig. 5). The model samples of a mixture of 60% blood content and 40% sodium citrate solution (5% solution, which was used for syringe washing) were studied. It turned out that the addition of sodium citrate to whole blood practically does not affect the position of the 2,3-diphosphoglycerate lines, and slightly increases the chemical shifts of inorganic phosphate lines. This is evident, as sodium citrate is used as a buffer compound to prevent pH change in cells. In really analyzed

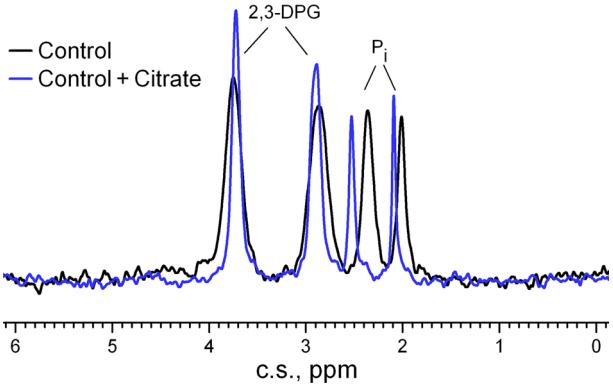

Fig. 5 Effect of sodium citrate on the ^{31}P NMR spectrum of blood

samples the sodium citrate concentration was much smaller, as it will be shown further, and its effect was much weaker.

For the purpose of additional characterization of whole blood samples its ^{1}H NMR spectra were also studied (Fig. 6). The ^{1}H NMR spectra of whole blood without adding any substances to it were not very informative because of high viscosity of the samples and evidently small amount of blood. In ^{1}H NMR spectrum (Fig. 6) the most intensive lines are the line of water and the lines of free EDTA-anticoagulant, from standard ampoules for blood sampling which chemical shift corresponds to data [18, 19]. Together with these lines less intensive sodium citrate lines were also recorded similar to [19, 20], they were an indicator of the trace amount of this blood preservative in the samples. Based on their amplitude we determined that the concentration of sodium citrate in the samples was extremely low and did not exceed 0.5%. As shown in [18], two anticoagulant substances referred here do not affect the

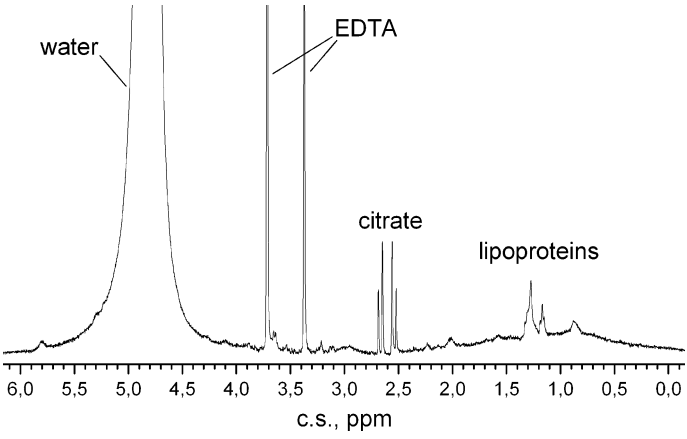

Fig. 6 Typical ^{1}H NMR spectrum of the rat blood

state of blood metabolites. In ^{1}H NMR spectra we also revealed some very weak lines in the region of chemical shifts corresponding to lipoproteins. However, it was impossible to quantify their amount without additional long accumulation.

3 Discussion

When evaluating the parameters of ^{31}P NMR spectra, the characteristics of six signals were considered. The chemical shift value and the integrated intensity of each line were obtained and statistically processed, where it was possible. The average values calculated for each line in two groups of rats are shown in Table 1. The transformation of ^{31}P NMR spectra of blood after 3-day spinal cord injury in rats is shown in Fig. 3.

The comparison of ^{31}P NMR blood spectra (Fig. 3) and their parameters (Table 1) in control rats and rats with SCI revealed quite significant difference between them, and made it possible to set some patterns. The most noticeable effects were an increase in line intensities and a decrease in the chemical shift values in ^{31}P NMR spectra of blood after SCI. The found differences can be characterized more detailed in the following way.

1. ^{31}P NMR spectra in blood of injured rats demonstrated a significant increase in intensity of signals 1 and 2 from ^{31}P nuclei in 2,3-diphosphoglycerate molecules (Figs. 3, 7). On average the integrated intensity in injured rats was 40% greater compared to the control group, that indicated the increase in a number of 2,3-diphosphoglycerate molecules.

2,3-diphosphoglycerate is the most abundant organic phosphate compound in erythrocyte, its concentration depends on the amount of inorganic phosphate—Pi,

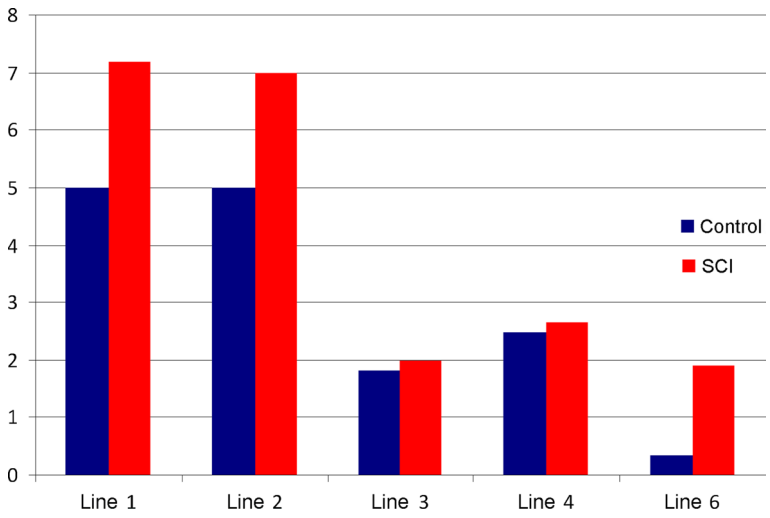

Fig. 7 Quantitative changes in the integrated intensities of ^{31}P NMR lines in rat blood after SCI

which is a cofactor of erythrocyte glycolysis. The main function of 2,3-DPG is hemoglobin-O_2 affinity regulation. A molecule of 2,3-DPG binds to deoxyhemoglobin stabilizing the low affinity (T) conformation. This way the number of 2,3-DPG actually characterizes the amount of deoxyhemoglobin. It is known that with a lack of oxygen in the medium the concentration of 2,3-DPG in erythrocytes increases, as a result the affinity of hemoglobin for oxygen decreases, the oxygen dissociation curve shifts to the right, and the oxygen supply to tissues increases. The regulation of the 2,3-DPG synthesis in erythrocytes is one of the main adaptation mechanisms facilitating the transport of oxygen under conditions of hypoxia [21]. Thus, the amplitude of 2,3-DPG peaks in NMR spectra correlates with the hypoxia level in peripheral tissues and the observed increase in signals 1, 2 indicates the increase in hypoxia after SCI.

2. For all intensive ^{31}P NMR lines 1–4 in the blood samples of injured rats a displacement toward lower chemical shift values was a characteristic feature (Fig. 3). The decrease of the chemical shift of the lines 1–4 varied from 0.1 to 0.4 ppm. As the position of the 2,3-DPG lines depends on pH, the shift of the lines 1, 2, 4 after SCI indicates a decrease in pH within the erythrocyte, and the shift of the line 3 indicates a decrease in pH in blood plasma [1]. In general, pH change characterizes the acidification of the chemical medium, i.e., acidosis. In this study the average position of line 1 was shifted from 3.72 ppm (in control group) to 3.60 ppm (in injured group), and the average position of line 2 from 2.85 ppm to 2.71 ppm, respectively. The averaged shift of lines 1 and 2 due to the SCI was 0.12 and 0.14 ppm.

The dependence of chemical shifts of 2,3-DPG signals on pH level in whole blood, erythrocytes and hemolysate was studied in [1] and a method for evaluating the intracellular pH in erythrocyte by the chemical shift of lines 1 and 2 or by relative difference of chemical shifts between 2,3-DPG lines was proposed. The use of this technique allowed us to estimate pH value inside the erythrocytes.

Using referred dependences on pH (Figs. 2 and 5 in [1]) we estimated the change in pH level inside the erythrocytes after SCI. The described above changes in chemical shifts of lines 1 and 2 corresponded to the decrease in pH by 0.1; within the erythrocytes of control rats pH value was ~ 7.2, while in rats with SCI it was ~ 7.1.

According to [1], the difference between positions of lines 1 and 2, denoted as Δ, also depends on pH [1]. In rat blood of control group averaged Δ value was 0.87 ppm, and in blood of injured rats this difference was 0.89 ppm. Δ value change also illustrates the decrease in pH inside the erythrocyte (Fig. 2 in [1]).

3. Along with changes in the intensity of signals 1, 2 an increase in intensity of signals 3, 4 of inorganic phosphates was observed. It can be evidence of the intensification of dephosphorylation processes of organic molecules in a chemical medium with a reduced pH. In the blood of injured rats 8–9% increase of inorganic phosphates was observed (Fig. 7). This fact is consistent with the results of clinical studies of blood plasma in patients with SCI being examined during the first 3 months after injury [22]. The study revealed the increase in the amount of phosphorus as well as alkaline phosphatase which catalyzes the cleavage of phosphate from many types of molecules.

4. In some blood samples after SCI a weak line 5 due to AMP, the product of the ATP decay arise. It is known that in acidic medium dephosphorylation of ATP

occurs, that leads to adenosine diphosphate (ADP) formation, with subsequent dephosphorylation to the form of AMP and inorganic phosphates. This explains the appearance in the blood of rats with SCI the weak signal from AMP. Referred processes may be also the reason of amplification of Pi signals (lines 3, 4).

5. In the blood of injured rats phospholipid signals (line 6) emerged, which were usually absent in control rats. The increasing of ^{31}P NMR phospholipid signal in injured rat blood agrees with the results of in vivo ^{31}P NMR studies of spinal cord in animals with SCI and without it. Using in vivo ^{31}P NMR the authors of [12] observed in intact spinal cord the wide ^{31}P NMR signal corresponded to myelin phospholipids. And in [13] it was found, that after SCI this signal in spinal cord was rather decreasing giving evidence for degradation of nerve cells membranes, the reduction of intracellular pH was also revealed in SCI [13]. The acidification observed in the pathology of the spinal cord enhances free radical processes and can cause the neuronal death. Thus, the appearance of phospholipid signal after SCI in the blood may be caused by the release of the membrane phospholipids out of the destroyed cellular structures of spinal cord. At the same time, one can not rule out the process of phospholipid release from the membranes of erythrocytes destructed under conditions of hypoxia and acidosis.

In addition, the weak lines from α, β, γ-phosphorus nuclei in the ATP molecule were recorded in ^{31}P NMR spectra of the blood samples (Fig. 4). But the intensities of these lines and the number of ATP molecules in control samples and SCI samples were not estimated, on the reason of low signal-to-noise ratio. At the same time, a small shift of ATP lines at ~ 0.25 ppm was observed, evidently due to pH change in blood after SCI.

According to the results of this work, an increase of the amount of 2,3-DPG in the blood of rats was observed 3 days after the spinal cord injury, which is apparently an adaptive response to the increasing hypoxia in tissues under the given conditions. It is known that moderate hypoxia is one of the factors which lead the body into a state of increased mobilization. Various studies have showed numerous changes that could be attributed to compensatory-adaptive. In particular, it is known that hypoxia can affect the production of nitric oxide. If strong hypoxia leads to suppression of NO production, then in moderate hypoxia there is a compensatory mechanism increasing NO production. Earlier, such results were obtained by EPR spectroscopy in rats after SCI [23]. They showed that generalized activation of the nitric oxide system was observed on the 3rd day after spinal cord injury and NO production increased not only in damaged spinal cord tissue, but also in blood, liver and heart tissues. The precise mechanisms of compensatory rearrangements under conditions of hypoxia are not completely understood. In [24] the key role of red blood cells in sensitivity to hypoxia was determined. The increase of the number of 2,3-DFG in the blood after a spinal cord injury is one of the ways to increase the supply of oxygen to tissues under hypoxic conditions, by the affecting the hemoglobin affinity to oxygen. By inducing of the synthesis of vasoactive metabolites, such as NO, the supply of oxygen from blood to the tissues increases due to vasodilation. These paths, apparently, intersect and interact with each other.

4 Conclusion

As the result of ^{31}P NMR study of blood at SCI condition, the organic and inorganic phosphorus compounds in blood, such as: 2,3-diphosphoglycerates, inorganic phosphates, phospholipids, AMP, ATP were characterized.

As the level of 2,3-DPG effects the affinity of hemoglobin for oxygen, so the observed increase in intensity of ^{31}P NMR signals of 2,3-DPG after SCI demonstrates the shift of the oxygen dissociation curve to the right and consequently the increase of oxygen supply to tissues in conditions of hypoxia. These signals are an indicator of hypoxia.

The ^{31}P NMR investigation of whole blood detected pH decrease at SCI condition both inside the erythrocytes and in plasma.

Phospholipids were detected in blood of injured rats. Small amount of AMP in blood after SCI indirectly testifies the intensification of ATP dephosphorylation in condition of acidosis.

Thus, the information obtained by ^{31}P NMR spectroscopy allowed to receive the new data about the mechanisms of changes in phosphorus metabolism after spinal cord injury not available for other methods. The observed increase in 2,3-DPG and activation of the nitric oxide production system after a spinal cord injury indicate compensatory-adaptive changes under conditions of hypoxia and acidosis, and the ^{31}P NMR signals of phospholipids may be used as an indicator of the degree of destruction of cellular structures.

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