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# Changes in the Content of Neuroglobin in the Neurons of the Cerebral Cortex of Rats with Ischemia

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ABSTRACT

**Introduction:** Neuroglobin is a metalloprotein of the family globin, which contains protoporphyrin with an iron atom in the center, forming six coordination bonds. Due to its ability to bind oxygen, Ngb enhances the supply of oxygen to the mitochondria of metabolically active neurons.

**Objectives:** To assess the content of neuroglobin in the brain of rats with cerebral ischemia of varying severity.

**Materials and methods:** The experiments were performed on 60 male outbred white rats weighing 260±20 g in compliance with the requirements of the Directive of the European Parliament and of the Council No. 2010/63/EU of September 22, 2010 on the protection of animals used for scientific purposes. Modeling of the cerebral was carried out under conditions of intravenous thiopental anesthesia (40-50 mg/kg). The studies used models of total, subtotal, stepped subtotal, and partial cerebral ischemia.

**Conclusion:** The most pronounced disorders of the prooxidant-oxidant balance, as well as a decrease in the content of neuroglobin, were observed during cerebral ischemia lasting 1 day. During cerebral ischemia with ligation of both common carotid arteries with an interval of 7 days, an increase in the content of neuroglobin was noted, as a manifestation of the activation of compensatory mechanisms.

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## Introduction

With cerebral ischemia (CI), a chain of pathogenetic disorders develops in its structures, among which one of the leading ones is energy deficiency, which leads to the development of cellular pathology due to disturbances in homeostasis, enzyme activity, membrane integrity, and the operation of energy pumps of ion channels. Under the conditions of CI, the mechanisms of synaptic transmission are selectively disrupted, which contributes to disruption of autoregulation of local blood flow, the development of vasospasm, increased platelet aggregation and the development of intravascular stasis, which deepen hypoxia and increase energy deficiency. The work of enzymes, including sodium-potassium ATPase, is disrupted, leading to an imbalance of ions and cerebral edema [1,2].

Neuroglobin (Ngb) is a metalloprotein of the family globin, which contains protoporphyrin with an iron atom in the center, forming six coordination bonds. Ngb is predominantly expressed in the nervous system, retina, and some endocrine structures [1]. The similarity of the structure of Ngb with other globin proteins [2] suggests similar functions of providing oxygen homeostasis of cells [4]. But since the level of Ngb affinity for oxygen is very high, and this is an obstacle to the release of oxygen, this metalloprotein is increasingly considered as an indicator of the level of oxygen in mitochondria, which oxidize organic substances and form ATP [6]. At the cellular level, Ngb is found in the cytoplasm, vesicular structures, neurotubules, nucleus, and mitochondria [4]. Due to its ability to bind oxygen, Ngb enhances the supply of oxygen to the mitochondria of metabolically active neurons. This hypothesis is supported by the existence of Ngb in metabolically active cells and subcellular compartments.

The role of Ngb in hypoxia/ischemia is still unclear. Some studies indicate its neuroprotective effect in cerebral ischemia due to

increased expression of endothelial NO synthase. Other data refute its significance for the survival of neurons in oxygen deficient conditions, since Ngb deficiency appears to increase HIF-1α expression. Thus, neuroglobin, according to the literature, can act as a neuroprotector in cerebral ischemia, but its content in brain neurons during ischemia of varying severity remains unexplored.

**Objectives**

The purpose is to assess the content of neuroglobin in the brain of rats with cerebral ischemia of varying severity.

**Materials and Methods**

The experiments were performed on 60 male outbred white rats weighing 260±20 g in compliance with the requirements of the Directive of the European Parliament and of the Council No. 2010/63/EU of September 22, 2010 on the protection of animals used for scientific purposes. Modeling of the cerebral was carried out under conditions of intravenous thiopental anesthesia (40-50 mg/kg).

The studies used models of total (TCI), subtotal (SCI), stepped subtotal (SSCI), and partial (PCI) cerebral ischemia. Total cerebral ischemia (TCI) was modeled by decapitation of animals. The brain sampling was carried out 1 hour and 24 hours after decapitation - to study the parameters of the prooxidant-antioxidant balance, and also after 1 hour to determine the content of neuroglobin.
Subtotal cerebral ischemia (SCI) was modeled by simultaneous ligation of both common carotid arteries (CCA). The material was taken 1 hour and 24 hours after decapitation - to study the parameters of the prooxidant-antioxidant balance, and also 1 hour later to determine the content of neuroglobin.

Stepwise subtotal cerebral ischemia (SSCI) was performed by successive ligation of both CCA with an interval of 7 days (subgroup 1), 3 days (subgroup 2), or 1 day (subgroup 3). The material was taken 1 hour after ligation of the second CCA in each of the subgroups. Partial cerebral ischemia (PCI) was modeled by ligation of one CCA on the right. The material was taken 1 hour after the operation.

Determination of the content of neuroglobin was carried out by immunohistochemical method using monoclonal antibodies. For this purpose, after decapitation, the brain was quickly removed from rats, pieces of the cerebral cortex were fixed in zinc-ethanol-formaldehyde at +4°C (overnight), then embedded in paraffin. Paraffin sections 5 μm thick were prepared using a microtome and mounted on glass slides. The preparations were processed according to the protocol of immunocytochemical reaction for light microscopy, excluding the procedure of thermal unmasking of antigens. To determine the immunoreactivity of the molecular marker of neuroglobin, primary monoclonal mouse Anti-Ngb antibodies from Abcam (Great Britain, ab. 14748) were used at a dilution of 1:600 at +4°C, exposure 20 h, in a humid chamber [3]. The EXPOSE Mouse and Rabbit specific HRP/DAB detection IHC kit Abcam (UK, ab. 80436) was used to detect bound primary antibodies. The content of neuroglobin was studied in the cytoplasm of neurons of the fifth layer of the parietal cortex and neurons of the field CA1 of the hippocampus in immunohistochemical preparations based on the optical density of the chromogen sediment using an Axioscop 2 plus microscope (Zeiss, Germany), a digital video camera (LeicaDFC 320, Germany) and ImageWarp image analysis program (Bitflow, USA).

To prevent a systematic measurement error, brain samples from the compared control and experimental groups of animals were studied under the same conditions. As a result of the research, quantitative continuous data were obtained. Since the experiment used small samples that had a non-normal distribution, the analysis was performed by nonparametric statistics using the licensed computer program Statistica 10.0 for Windows (StatSoft, Inc., USA). The data are presented as Me (LQ; UQ), where Me is the median, LQ is the value of the lower quartile; UQ is the value of the upper quartile. Differences between groups were considered significant at $p<0.05$ (Kruskell-Wallis test with Bonferoni correction).

**Results**

In TCI, there was a decrease in the content of neuroglobin involved in the binding and neutralization of reactive oxygen species, compared with the control - by 69 (62; 73)% in the parietal cortex, $p<0.05$ and by 68 (64; 75)% in hippocampus, $p<0.05$ (table 1, figures 1, 2). At the same time, there were no differences in the content of neuroglobin in the parietal cortex and hippocampus ($p>0.05$).

Compared with the control, in the SCI group, the content of neuroglobin decreased by 32(29;39)% in the parietal cortex ($p<0.05$) and by 29(22;34)% in the hippocampus ($p<0.05$). Compared to the TCI group, the content of neuroglobin in rats with SCI was higher in the parietal cortex by 55(49;62)%, $p<0.05$ and in the hippocampus by 56(48;61)%, $p<0.05$. The decrease in the content of neuroglobin in rats with SCI was less pronounced than in rats with TCI - by 23% in the parietal cortex ($p<0.05$) and by 27% in the hippocampus ($p<0.05$). In rats with PCI, there was no change in the content of neuroglobin in relation to the level in the control group ($p>0.05$).

At the same time, compared with the TIGM group, the content of neuroglobin in the cytoplasm of neurons was higher by 69(62;75)% in the parietal cortex ($p<0.05$) and by 69(63;76)% in the hippocampus ($p <0.05$), and compared with the SCI group - by 32(26;38)%, $p<0.05$ and by 28(21;34)%, $p<0.05$, respectively. Compared with the "control" group, in the 1st subgroup of SSCI (the interval between dressings was 7 days), there was an increase in the content of neuroglobin by 13(9;17)% in the parietal cortex ($p<0.05$) and by 14(8; 19)% - in the hippocampus ($p<0.05$), in the 2nd subgroup of SSCI (interval between dressings 3 days) the content of neuroglobin decreased - by 13(7;16)% in the parietal cortex ($p<0.05$) and by 7(4;12)% in the hippocampus ($p<0.05$), and in the 3rd subgroup, the greatest decrease in the content of neuroglobin was noted - by 31(26;39)%. $p<0.05$ and by 33(27;43)%, $p<0.05$, respectively.

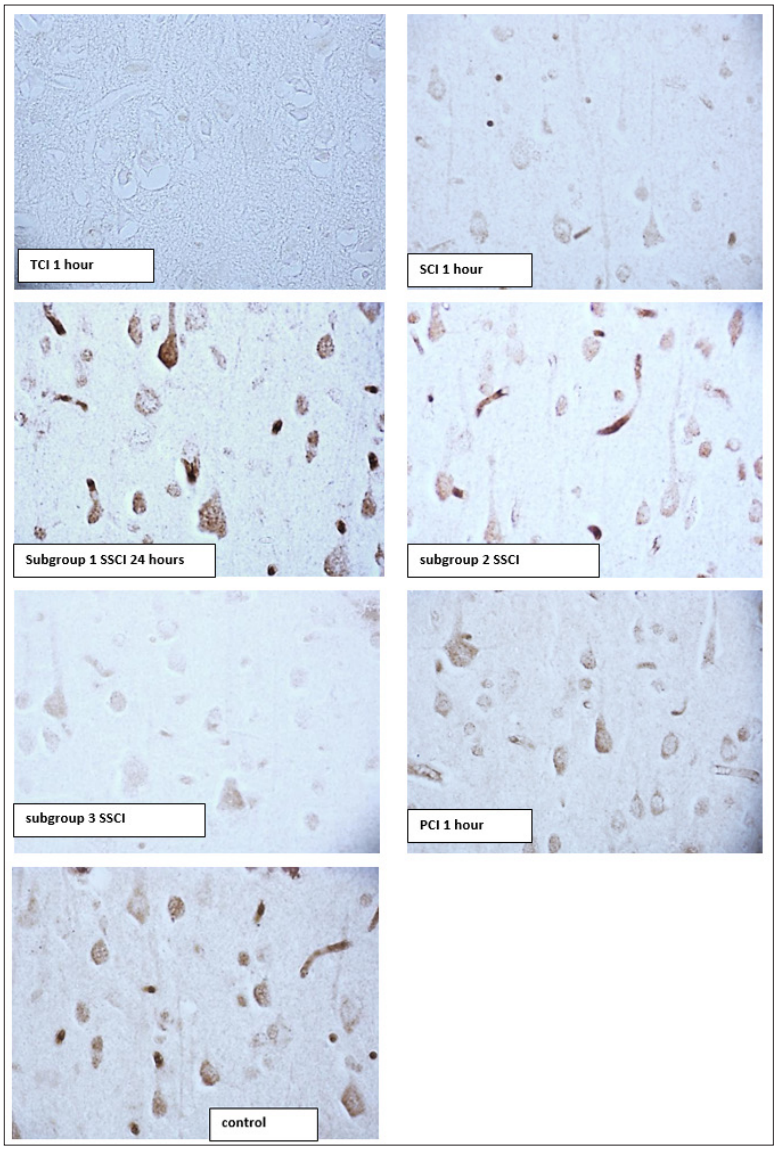
In the 3rd subgroup of SSCI (the interval between dressings of the CCA is 1 day), the content of neuroglobin was lower compared to the 1st subgroup by 40(35;46)% in the parietal cortex ($p<0.05$) and by 43(36 ;49)% - in the hippocampus ($p<0.05$), and compared with the 2nd subgroup of SSCI - by 21(16;25)%, $p<0.05$) and by 28(17;33)%, $p<0.05$, respectively. Thus, the least pronounced decrease in the content of neuroglobin was observed in the 1st subgroup of SSCI with an interval between dressings of 7 days, while the largest was observed in the 3rd subgroup with a minimum interval between dressings of the CCA (1 day).

Compared to SCI, modeled by simultaneous ligation of both CCA, no differences in the content of neuroglobin in the cytoplasm of neurons were found in the 3rd subgroup of SCI in both studied departments ($p>0.05$). The content of neuroglobin in the 2nd subgroup of SBI was 21(18;28)% higher than in SCI in the parietal cortex ($p<0.05$) and 24(19;29)% in the hippocampus ($p<0.05$) and in the 1st subgroup SSCI - by 40(32;48)%, $p<0.05$ and by 39(31;47)%, $p<0.05$, respectively.

**Table 1: The Content of Neuroglobin in the Cytoplasm of Pyramidal Neurons of the Parietal Cortex and Field Ca1 of the Hippocampus of the Brain of Rats with Cerebral Ischemia, Me (Lq; Uq)**

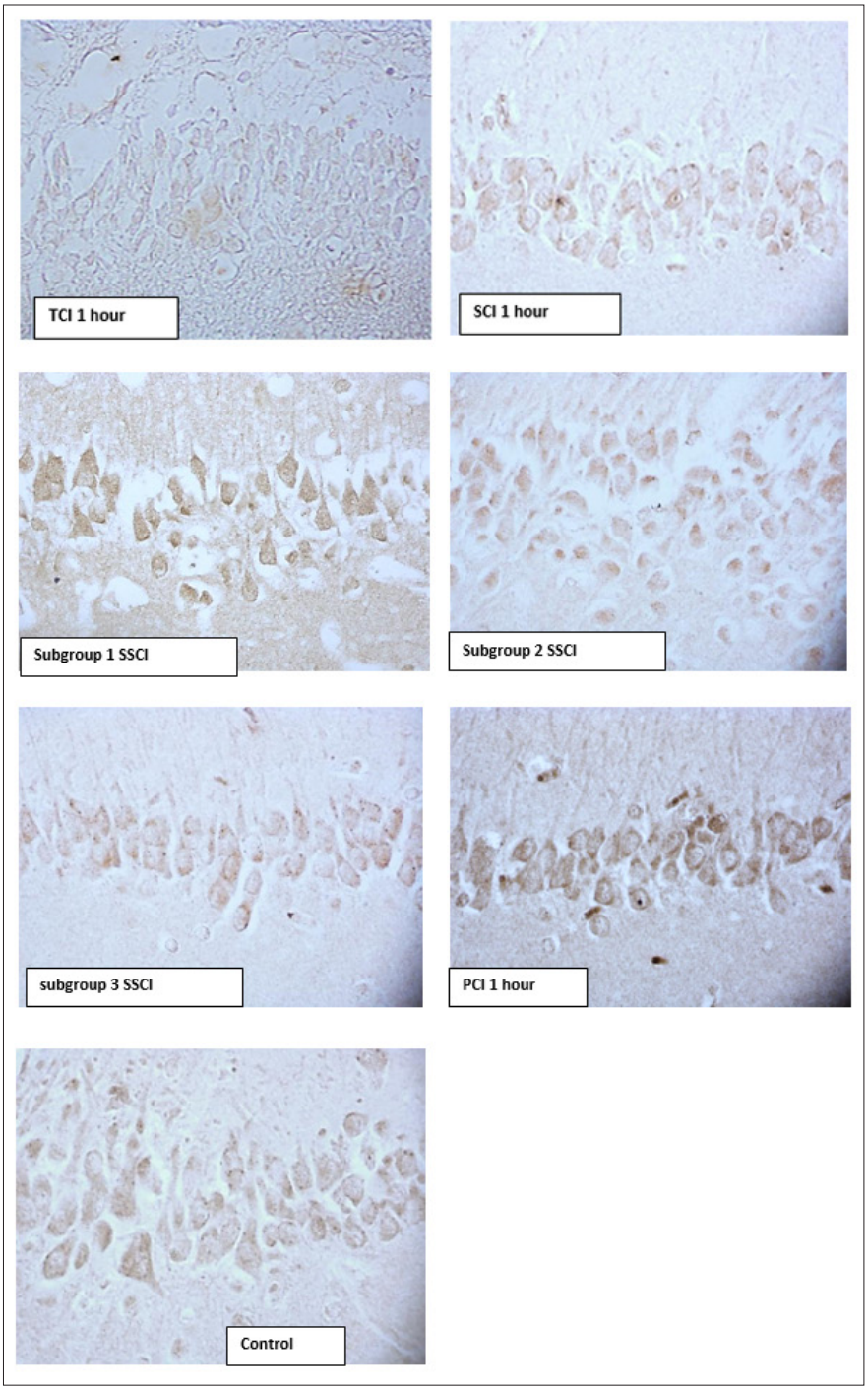
| Groups | | The content of neuroglobin / units of optical density | |
|---|---|---|---|
| | | Parietal cortex | Hippocampus |
| Control | | 0,167(0,162;0,172) | 0,165(0,163;0,165) |
| TCI | | 0,051(0,049;0,052)* | 0,052(0,049;0,054)* |
| SCI | | 0,114(0,108;0,116)* | 0,117(0,107;0,126*) |
| PCI | | 0,166(0,163;0,175) | 0,162(0,158;0,166) |
| SSCI | 1 subgroup | 0,191(0,186;0,193)* | 0,192(0,191;0,216)* |
| | 2 subgroup | 0,145(0,142;0,152)* | 0,153(0,149;0,158)* |
| | 3 subgroup | 0,115(0,111;0,123)* | 0,111(0,108;0,117) |

Notes
TCI – total cerebral ischemia
SCI – subtotal cerebral ischemia
SSCI – stepped subtotal cerebral ischemia
PCI – partial cerebral ischemia
Notes: * – p<0,05 compared to the control group




Digital micrograph. Zoom 40.

**Figure 1:** the Content of Neuroglobin in the Cytoplasm of Pyramidal Neurons of the Parietal Cortex of Rats with Total (TCI), Subtotal (SCI), Stepped Subtotal (SSCI) and Partial Cerebral Ischemia (PCI)




Digital micrograph. Zoom 40.

**Figure 2:** The content of neuroglobin in the cytoplasm of the pyramidal neurons of field CA1 of the hippocampus of rats with total (TCI), subtotal (SCI), stepped subtotal (SSCI) and partial cerebral ischemia (PCI)

Compared with the "PCI" group, in the 1st subgroup of SSCI, the content of neuroglobin was 13 (8; 18)% higher in the parietal cortex ($p < 0.05$) and 16(10; 24)% in the hippocampus ($p < 0.05$). In the 2nd subgroup of SSCI, it was less by 13(9;17)% in the parietal cortex ($p<0.05$) and by 12(9;22)% in the hippocampus ($p<0.05$), and in the 3rd subgroup of SSCI, the decrease in the content of neuroglobin was more pronounced and amounted to 31(25;37)% ($p<0.05$) and 28(23;33)% ($p<0.05$), respectively.

Thus, the changes in the content of neuroglobin in the 1st, 2nd and 3rd subgroups of SSCI were multidirectional: in the 1st subgroup with a maximum interval between CCA dressings of 7 days, it increased, indicating the activation of compensation mechanisms during hypoxia by increasing the deposition and oxygen transfer to the mitochondria of neurons, while as the time interval between CCA ligations shortened, its content decreased, indicating insufficient inclusion of compensation mechanisms in more severe forms of CI.

The content of neuroglobin in the 1st subgroup of SSCI (the interval between dressings of the CCA was 7 days) was closest to the value of the indicators in the group "PCI" and "control", while in the 3rd subgroup of SSCI, with a minimum interval between dressings of the CCA 1 day, to a greater extent was close to the values of the protein content in the "SCI" group, modeled by simultaneous ligation of both CCAs, which indicates a more pronounced violation of antihypoxic protection.

With SCI with ligation of both CCA with an interval of 7 days, an increase in the content of neuroglobin was noted, as a manifestation of the activation of compensatory mechanisms: an increase in the efficiency of the processes of utilization of oxygen and oxidation substrates and their delivery to the mitochondria of neurons due to the effects of neuroglobin, an increase in the synthesis of nucleic acids and proteins , transport of O2 and metabolic substrates, the dominance of the activity of anabolic processes over catabolic [6], which reduces the severity of oxidative stress.

An increased content of neuroglobin in the cytoplasm of neurons in the 1st subgroup of SSCI with ligation of both CCA with an interval of 7 days can also help optimize oxygen supply to mitochondria. In addition, neuroglobin is a free radical scavenger, which contributes to the normalization of the prooxidant-antioxidant balance of the brain at the free-radical stage. Thus, the most pronounced disorders of the prooxidant-oxidant balance, as well as a decrease in the content of neuroglobin, were observed during total CI lasting 1 day. Similar, however, less pronounced disorders were found in daily SCI and in the subgroup of SSCI with an interval between ligation of the CCA of 1 day. When modeling partial CI due to compensation of blood circulation in the circle of Willis, there were no pronounced changes in the content of neuroglobin, while non-simultaneous ligation of the CCA with an interval of 7 days led to an increase in the content of neuroglobin.

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