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Original article

Evaluating some parameters of Wistar rat brain in traumatic brain injury model with administration of proline-containing peptides

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Abstract: *Objective* — To examine the morphometric parameters of neurons and the oxidative status of the Wistar rat brain tissues after administering proline-containing peptides, also known as glyprolines (Arg-Gly-Arg-Pro-Gly-Pro [RGRPGP] and Thr-Lys-Pro-Arg-Pro-Gly-Pro [WKPRPGP; Selank]), on a traumatic brain injury (TBI) model.

Study subjects — Our study involved 26 mature male Wistar rats (2.5-3 mos. old, 220-300 g). The morphometric parameters of neurons and the oxidative status of animal brain tissues were studied.

Material and Methods — Four experimental groups were formed. Group 1 included intact control animals. Animals in three other groups were subjected to TBI via free fall of a 50 g weight from a height of 50 cm on the second day of the experiment and received the following injections: 0.9% sodium chloride solution in Group 2; WKPRPGP peptide solution in Group 3; RGRPGP peptide solution in Group 4. Substances were administered intraperitoneally on a daily basis at a dose of 0.1 mg/kg from day 1 through day 5 of the experiment. Morphometric parameters of rat brain neurons were studied on paraffin sections stained with hematoxylin and eosin. The intensity of free radical processes in the brain tissue was investigated by chemiluminescence.

Results — An analysis of morphometric parameters revealed significant increases in the neuronal cytoplasm area, nucleolar area, number of nucleoli, and nuclear-nucleolar index with the introduction of the RGRPGP peptide after TBI, compared with the WKPRPGP peptide under the same conditions. After TBI and peptide administration, we observed an oxidative stress in the neocortex of Wistar rats, and it was more pronounced in the group of animals treated with RGRPGP.

Conclusion — After RGRPGP peptide administration, we observed an increase in the morphometric parameters of neurons in the closed TBI model: a larger area and a greater number of nucleoli. Chemiluminescence data implied that WKPRRPGP peptide better protected brain tissue in rats from the effects of oxidative stress caused by TBI.

Keywords: glyprolines, traumatic brain injury, neocortex, oxidative status.

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Introduction

Traumatic brain injury (TBI) triggers a cascade of post-traumatic stress reactions, often leading to harmful changes in the central nervous system [1]. The search for medications that would reduce the risk of undesirable effects is ongoing. Regulatory peptides, compounds with multifaceted biological activity, performed well in combating stress of various origins [2, 3]. Hence, studying new compounds of this group and comparing them with oligopeptides registered as medicines is very relevant.

Study objective: Comparison of the effects of WKPRPGP peptide (also known as the registered medicine Selank) and RGRPGP peptide on the morphometric parameters and oxidative status of the brain tissues in sexually mature Wistar males after intraperitoneal administration of the peptide solution after TBI.

Material and Methods

Study subjects

In the experiment, we used Wistar male rats at the age of 90 days (n=26). The animals were kept in a vivarium at a temperature of 22-24 °C with *ad libitum* access to food and water. They were fed standard granulated chow for laboratory rodents.

Tested substances

WKPRRPGP and RGRPGP peptides were synthesized at the Institute of Molecular Genetics of the Russian Academy of Sciences and provided by Academician N.F. Myasoyedov.

Experimental design

The design of our study was planned in compliance with the requirements of the *Rules of Laboratory Practice in the Russian Federation* (Order of the Ministry of Healthcare of the Russian

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Federation No. 708-n of August 23, 2010) and the *European Union Directive on the Protection of Animals Used for Scientific Purposes* (2010/63/EU). Keeping animals in captivity and their removal from the experiment was performed in accordance with the law *On Protecting Animals from Cruelty* (Chapter V, Article 104679-GD of December 01, 1999). The study protocol was approved at a meeting of the Ethics Committee at the Far Eastern State Medical University of the Russian Ministry of Healthcare (Minutes No. 2 of February 5, 2019).

The allocation of animals into groups was carried out two weeks prior to the start of the experiment. During the adaptation period and in the course of the experiment, the animals were weighed daily. The following experimental groups were formed:

1. Control group (n=8): (intact animals);
2. NaCl group (n=6): administering 0.9% sodium chloride solution in equivoluminous doses for 5 days after TBI;
3. Selank group (n=6): administering Thr-Lys-Pro-Arg-Pro-Gly-Pro peptide at a dose of 0.1 mg/kg for 5 days after TBI;
4. RGRPGP group (n=6): administering Arg-Gly-Arg-Pro-Gly-Pro peptide at a dose of 0.1 mg/kg for 5 days after TBI.

On day 1 of the experiment, the animals were subjected to a simulated closed TBI (except for the animals of the control group) of mild severity (TBI resulting from a fallen load) [4]. Animals received their first injection two hours after TBI.

From day 2 through day 5 of the experiment, in the morning, the animals were injected intraperitoneally with a relevant peptide at a dose of 0.1 mg/kg (Selank and RGRPGP groups), or with equivoluminous dose of 0.9% sodium chloride solution (NaCl group).

On day 6, the animals were removed from the experiment. Rats were euthanized by decapitation under inhalation anesthesia with chloroform vapors. Then their brain was removed, the right hemisphere of the brain was fixed in a zinc-containing fixative (zinc-ethanol-formaldehyde) [5]. The gray matter of the left hemisphere of the brain was taken for examining the free radical status of the tissue.

Table 1. Morphometric parameters of neurons in 90-day old Wistar rats in the studied groups (M±m). The second layer of anterior parietal lobe

Morphometric parameters	*Control (n=8)*	*NaCl (n=6)*	*Selank (n=6)*	*RGRPGP (n=6)*
Neuron body area (μm2)	102.6±10.1	91.4±5.1	85.4±8.8 @p<0.01	105.1±10.6 *p<0.01
Nucleus area (μm2)	58.2±4.8	54.9±5.3	55.8±6.1	62.4±6.3 &p=0.03
Total area of the nucleoli (μm2)	4.7±0.5	4.3±0.3	4.4±0.5	5.6±0.5 &p<0.01 @p<0.01 *p<0.01
Number of nucleoli	1.0±0.05	1.1±0.1	1.1±0.07	1.1±0.1
NCR	0.6±0.03	0.6±0.02	0.6±0.01 @p<0.01	0.6±0.02
NNR	0.08±0.01	0.08±0.007	0.08±0.01	0.09±0.009

* – Significant differences with the Selank group; # – significant differences with RGRPGP group; & – significant differences with NaCl group; @ – significant differences with control group; n – number of animals in the group. NCR, nuclear-cytoplasmic ratio; NNR, nucleolar-nuclear ratio.

Table 2. Morphometric parameters of neurons in 90-day old Wistar rats in the studied groups (M±m). The second layer of main parietal lobe

Morphometric parameters	*Control (n=8)*	*NaCl (n=6)*	*Selank (n=6)*	*RGRPGP (n=6)*
Neuron body area (μm2)	88.1±32.8	100.5±11.7	84.7±8.0 &p=0.02	99.9±12.4 *p=0.05
Nucleus area (μm2)	49.0±18.3	60.0±6.7	55.1±5.9	61.1±6.6 @p=0.04
Total area of the nucleolus (μm2)	3.8±1.4	4.5±0.8	4.1±0.7	5.4±0.04 @p<0.01 * p<0.01
Number of nucleoli	1.1±0.05	1.2±0.06	1.0±0.03 &p< 0.01 @ p< 0.01	1.1±0.07 *p=0.015
NCR	0.6±0.03	0.6±0.02	0.6±0.03	0.6±0.01
NNR	0.08±0.01	0.08±0.01	0.07±0.008	0.1±0.008 *p<0.01 @p=0.03 &p=0.03

* – Significant differences with the Selank group; # – significant differences with RGRPGP group; & – significant differences with NaCl group; @ – significant differences with control group; n – number of animals in the group. NCR, nuclear-cytoplasmic ratio; NNR, nucleolar-nuclear ratio.

Table 3. Morphometric parameters of neurons in 90-day old Wistar rats in the studied groups (M±m). The fifth layer of anterior parietal lobe

Morphometric parameters	Control (n=8)	NaCl (n=6)	Selank (n=6)	RGRPGP (n=6)
Morphometric parameters	160.0±14.7	114.1±15.8 @p<0.01	144.2±15.9 &p=0.023	105.7±36.5 @p<0.01 *p=0.036
Neuron body area (μm2)	85.3±8.4	65.96±8.6 @p<0.01	81.9±10.01 &p=0.023	85.1±54.2
Nucleus area (μm2)	6.2±0.5	5.7±0.6	6.1±0.3	5.3±2.3
Total area of the nucleolus (μm2)	1.0±0.1	1.2±0.01 @p<0.01	1.07±0.07	1.0±0.02 &p<0.01
Number of nucleoli	0.5±0.01	0.6±0.02	0.6±0.04	0.6±0,05
NCR	0.07±0.007 &p<0.035	0.09±0.01	0.07±0.004 &p<0.036	0.07±0.02 &p<0.04

* – Significant differences with the Selank group; # – significant differences with RGRPGP group; & – significant differences with NaCl group; @ – significant differences with control group; n – number of animals in the group. NCR, nuclear-cytoplasmic ratio; NNR, nucleolar-nuclear ratio.

Table 4. Chemiluminescence parameters of brain homogenates of 90-day old white Wistar rats in the studied groups (M±m)

Indicators (conventional units)	Control (n=8)	NaCl (n=6)	Selank (n=6)	RGRPGP (n=6)
S_{sp}	8.3±3.9	8.6±4.3	2.4±0,5 @p<0.01 &p<0.01	15.9±3.6 *p<0.01 @p<0.01 &p<0.01
S_{ind2}	57±13.4	22.5±11.9 @p< 0.01	11.9±4.6 @p< 0.01	34.6±5.7 @p< 0.01 *p<0.01

* – Significant differences with the Selank group; # – significant differences with RGRPGP group; & – significant differences with NaCl group; @ – significant differences with control group; S_{sp} – light intensity per 1 min of spontaneous chemiluminescence; S_{ind2} – light intensity per 2 min of H_2O_2-induced luminol-dependent chemiluminescence.

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For morphometric studies, after histological examination, we prepared 7 µm thick paraffin sections of the anterior parietal lobe (APL) and main parietal lobe (MPL), and stained them with hematoxylin and eosin [5]. Neuronal morphometry was performed on a MEKOS-Ts1 robotized microscope, including the following parameters: area of neuron bodies, nucleus area, the number and total area of nucleoli, nuclear-cytoplasmic ratio (NCR), and nucleolar-nuclear ratio (NNR) [2, 6]. The parameters were evaluated in five locales: in layers II and V of the APL and MPL, and in the CA1 region of the hippocampus. The parameters of 25 cells were analyzed in each locale (*Tables* 1–3).

The activity of free radical oxidation was determined in freshly prepared homogenates of the cerebral cortex sections via chemiluminescence (CHL) on the Perkin-Elmer LS-50B Luminescence Spectrometer (USA) sensu conventional laboratory methods [7, 8]. The signal was standardized using the built-in FinLab software. The following parameters were determined: S_{sp} (light intensity per 1 minute of spontaneous CHL that correlated with the intensity of free radical processes) and S_{ind2} (light intensity per 2 minutes of H_2O_2-induced luminol-dependent chemiluminescence that was inverse of the antioxidant and antiradical protection activity [7, 8]. The intensity of CHL was expressed in conventional units per 1 g of tissue (Table 4).

Statistical data processing

Statistical processing of experimental data was carried out using the MS Excel software program. After confirming the normality of the distribution of variational series, the values of the arithmetic mean (M) and standard deviation (SD) were determined. Comparisons between groups were performed using the nonparametric Mann-Whitney test. Differences were considered significant at $p \leq 0.05$ [9].

Results

Morphometric studies

We revealed no statistically significant differences in morphometric parameters in the fifth MPL layer and C1 region of the hippocampus between the groups.

In the second APL layer, the area of cell bodies was 1.2 times greater in the control group vs. Selank group. In both NaCl group and Selank group, the total area of nucleoli in rat brain neurons was 1.3 times smaller than in the RGRPGP group (*Table* 1).

In the second MPL layer, we observed a larger total area of nucleoli in NaCl group, compared with Selank group. The NNR value was 1.25 times lower in both control and NaCl groups, and 1.4 times lower in the Selank group than in RGRPGP group. The total area of nucleoli in Selank group was 1.3 times smaller than in the RGRPGP group. (*Table* 2).

In the fifth APL layer, the area of neuron bodies was greater in the control and Selank groups, compared with the RGRPGP group, by 1.5 and 1.4 times, respectively. The NNR value in the neurons of NaCl group animals was 1.3 times higher than in both Selank and RGRPGP groups (*Table* 3).

Studying indicators of antioxidant and antiradical protection

TBI significantly affects the free radical status of the neocortex in Wistar rats: statistically significant differences were detected for all parameters (*Table* 4).

In control group, compared with Selank group, a 3.5-fold increase in the intensity of free radical processes (S_{sp}) and a 4.8-fold reduction in the activity of antioxidant and antiradical protection (S_{ind2}) were observed. The level of activity of antioxidant and antiradical protection is inverse of S_{ind2} parameter.

When comparing NaCl and Selank groups, a 3.6-fold decrease in light intensity per minute of spontaneous CHL (S_{sp}) in the tissue of animals treated with the peptide was established.

Comparison of the oxidative status of control vs. RGRPGP groups yielded 1.9 times increase in the intensity of free radical processes (S_{sp}) and 1.6-fold increase and the activity of antioxidant and antiradical protection (S_{ind2}) in the latter group.

When comparing NaCl and RGRPGP groups, 1.8-fold increase in the intensity of free radical processes (S_{sp}) was registered.

In the Selank group, compared with the RGRPGP group, 6.6-fold reduction in the intensity of free radical processes (S_{sp}) and three-fold increase in the activity of antioxidant and antiradical protection (S_{ind2}) were detected.

Discussion

The medicine Selank is successfully used for treating brain damage of various origins. We compared the protective properties of the WKPRPGP peptide with less studied RGRPGP peptide. It is worth noting that the structure of the latter suggests properties similar to those of the former. Previously, differences in the effects of Selank (WKPRRPGP substance) were studied with single use, repeated use, and clinical course use. It has been proven that Selank did not cause adverse reactions and tolerance and did not have a withdrawal effect [3, 10]. Clinical trials of Selank confirmed its neuroprotective and anxiolytic effects, as well as its nonspecific activating effect, mainly on the frontal lobes of the cerebral cortex [11, 12].

The following morphometric characteristics of neurons were analyzed: the area of neuron bodies, the area of nuclei, the number of nucleoli and their size, NCR, and NNR. The size of nucleoli and their number directly depend on the synthesis of ribosomal RNA [13, 14]. A decrease in the area of both body and nucleus of neurons may indicate the consequences of stress [15]. A reduction in NCR, according to Krishop et al. [16], implied the long-term consequences of stress, including ischemia.

In the control group, we observed a larger relative area of nucleoli than in the NaCl group, which could imply a higher level of RNA synthesis. The area of bodies of the neurons in the second MPL layer of the NaCl group was larger than in neurons of the same layer in the Selank group. This may indicate a greater susceptibility to stress in animals treated with the peptide [2, 16].

The area of neuron bodies in the second APL layer, as well as other morphometric indicators (the area of the nucleus, the total area of the nucleoli) were the largest in the RGRPGP group, which could be indicative of a smaller stress effect and more intense RNA synthesis in neurons; this was also implied by a larger area and the number of nucleoli in the second MPL layer.

A.L. Yasenevskaya et al., while studying hepatocytes, obtained results similar to ours. They observed a decrease in the number of nucleoli under stress exposure and a compensatory protective increase in the number of nucleoli after the use of Selank [13]. Our results confirmed that the neuroprotective properties of the WKPRRPGP peptide (in Selank group) were comparable to the effects of the RGRPGP peptide. Comparing uses of the studied

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oligopeptides after TBI, we may assume an existence of the stimulation of compensatory repair mechanisms via minimizing the disruption of brain homeostasis and restoring the optimal microenvironment for neuronal recovery support [17].

According to some studies, morphological and functional disorders of neurons, glial cells and the vascular bed are observed already on the first day after TBI. The manifestations are variable and depend on the severity of TBI. On day 1, we could observe the disorders of hemodynamics and outflow of cerebrospinal fluid, and, consequently, a pronounced edema of cerebral tissues. At the cellular level, signs of apoptosis are observed, such as a change in the shape and color of cells, nuclei, loss of nucleoli, etc. On day 7 after TBI, the authors observed an aggravation of the edema, hemodynamics and CSF outflow disorders. Decline in the physiological activity of neurons even during treatment, and restoration of the brain macrostructure occurred within two weeks [18].

TBI leads to cell damage (neuroinflammation, neurodegeneration) and brain vessel damage. Proinflammatory mediators, secreted by activated glial cells (micro- and astroglia), neurons, and local population of mast cells, may contribute to secondary CNS damage after TBI. The molecular mechanisms of neurovascular pathology in TBI are still unclear. It is assumed that by increasing the production of interleukin-1, microglia can affect the functions of neurons. It is known that the brain has its own renin-angiotensin system, which plays an important role in the pathogenesis of neurodegenerative diseases. This regulatory system acts in the brain parenchyma through type 1 angiotensin II receptors, is activated in TBI, and contributes to continued brain damage. Besides, TBI causes a cascade of pathological reactions, including lipid peroxidation (LPO) [19].

It is known that the brain is most vulnerable to the action of free radicals and the development of oxidative stress, because the lipid content constitutes over 50% of the dry matter in the brain. Additionally, brain tissues are characterized by high metabolic rate and low rate of cell regeneration. The brain requires about 30% of the oxygen consumed by the body, and its tissues are highly sensitive to hypoxia and ischemic disorders [12]. Accordingly, an increased production of reactive oxygen species, along with a disorder of the antioxidant defense system, are among the leading mechanisms of cell damage in TBI [20].

In some studies, it was noted that the processes of secondary damage to the brain tissue, including those caused by LPO, prevail over the recovery processes [21]. Inhibition of peroxidation processes in brain tissues under the action of Selank (WKPRPGP substance) after stress was shown [2]. Earlier, on the basis of the Central Scientific Research Laboratory of the Far Eastern State Medical University, the effect of the RGRPGP peptide on the oxidative status of erythrocytes was studied in comparison with WKPRPGP after stress of various geneses. A more pronounced antioxidant effect was shown in the tissues of animals treated with WKPRPGP [8].

According to our data, the level of oxidative stress in the Selank group was the lowest, compared with other experimental groups.

After administration of RGRPGP peptide in the experiment with closed TBI, a deterioration in the free-radical status of brain tissues in Wistar rats, regarding S_{sp} and S_{ind2} parameters, was observed, compared with the values for animals in the control and NaCl groups.

Conclusion

In the experiment with closed TBI, the use of RGRPGP peptide, in contrast to WKPRPGP peptide, provided a correction of the morphometric parameters in neurons (cell area and number of nucleoli) up to the values in the control group.

According to chemiluminescence data, WKPRRPGP peptide better protected brain tissue in rats from the effects of oxidative stress caused by TBI.

Conflict of interest

The authors declare no conflicts of interest. The scientific research (publication of the article) was carried out with the financial support of the Public Procurement on the topic, “Participation of regulatory peptides of the glyproline series in maintaining tissue homeostasis under physiological conditions and in the development of pathology.”

Study limitations

Small samples of animals were used in our research, which led to the use of nonparametric criteria for assessing the statistical significance of differences in the studied parameters. Increasing sample sizes of experimental groups in further experiments may somewhat reduce an ambiguity of the results.

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