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The Oligopeptides, Homologous of $ACTH_{15-18}$ Sequence: Neurotrophic and Anti-inflammatory Activity on the Model of Cerebral Ischemia in Rats

Roman Danylovych Deiko[1, *], Serhiy Yuriyovych Shtrygol′[1], Tetyana Victorivna Gorbach[2], Alexandr Alexandrovich Kolobov[3], Andrey Semenovich Simbirtsev[3]

[1]Department of Pharmacology, National University of Pharmacy, Kharkiv, Ukraine

[2]Department of Biochemistry, Kharkiv National Medical University, Kharkiv, Ukraine

[3]Department of Chemistry of Peptides, State Research Institute of Highly Pure Biopreparations, St. Petersburg, Russia

Email address:

roman.deyko@gmail.com (R. D. Deiko), strygol@mail.ru (S. Y. Shtrygol′), v-gorbatch@yandex.ua (T. V. Gorbach), indolicidin@mail.ru (A. A. Kolobov), secretary@hpb-spb.com (A. S. Simbirtsev)

[*]Corresponding author

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Abstract: The series of tetrapeptides, homologues of $ACTH_{15-18}$ sequence that contain D-form of lysine and arginine have been synthesized at the State Research Institute of Highly Pure Biopreparations (Saint-Petersburg, Russian Federation). Previous research allowed to reveal their neuroprotective, anti-hypoxic and favorable psychotropic properties and capability to stimulate blood supply to the injured brain. The influence of neuropeptide on rats survival, neurological deficit (ND), nerve growth factor (NGF) expression, tumor necrosis factor α (TNF-α), interleukine-1 beta (IL-1β) and interleukine-4 (IL-4) levels in rats brain tissue was studied on the irreversible bilateral carotid occlusion model. Neuroprotective properties of the neuropeptides KK-1 and KK-5 namely increase in rats with acute ischemic stroke (AIS) survival rate up to 75% and 80%, respectively, were shown. Neuropeptides decreased ND 1.7 times compared with the untreated group (UG). Neuroprotective effect of the neuropeptides is manifested by their ability to decrease the level of NGF compensatory overexpression, pro-inflammatory and anti-inflammatory cytokines such as TNF-α, IL-1β, IL-4. It has been established that the mechanism of the tetrapeptides KK-1 and KK-5 neuroprotective activity is connected with their neurotrophic and anti-inflammatory action.

Keywords: Cerebral Ischemia, Cytokines, Nerve Growth Factor, Neuroprotection, Oligopeptides, Rats

1. Introduction

Rational therapy of the cerebral ischemia still remains an unsolved medical-social issue. Ischemic insult (II) occupies the first place among neurological diseases in industrialized countries according to the number of lethal cases. In the USA, every-year expenditures connected with insult accounts for more than 40 million dollars during last decade [1].

The most promising way for improvement of II medical treatment is using of the pathogenetic pharmacotherapy intended to ensure survival and normal functioning of the neurons around penumbra.

One of the key elements for the acute ischemic stroke (AIS) is inflammation in the nerve cells that attend with the following disturbance of their trophic activity [2]. It starts in a few hours after ischemic brain injury and reaches its maximal level in 12-36 hours [3]. Inflammation increases the blood-brain barrier permeability and activated leukocytes migration from the bloodstream to the ischemic area [4]. The activated leukocytes (granulocytes, monocytes/macrophages, lymphocytes) and also neurons and glial cells (astrocytes, microglia) synthetize specific pro-inflammatory and anti-inflammatory cytokines, expression of which correlates with a severity of cerebral ischemia [5]. These cytokines include

tumor necrosis factor α (TNF-α), interleukine-1 beta (IL-1β) and interleukine-4 among them. One of the compensatory mechanisms of neuronal functional activity during this phase is the overexpression of series of the neurotrophic factors, namely the nerve growth factor (NGF) [6]. The results of cerebral tissue ischemic catastrophe are the progressive neurological deficit (ND) and increasing of lethal case possibility. Elimination of the triggering phase from the series of events at the AIS favor to avoid the neuroapoptosis – leading factor of cell death by II, and also saves the penumbra cells.

According to the data mentioned above the medicine with neuroprotective activity should provide normalization of cytokines profile at the AIS, decrease overexpression of the neurotrophic factors and, as the result, reduce ND and increase survival rate.

Regulatory peptides as well as drugs developed on their basis are perspective class of the neuroprotectors. Numerous neuroprotective peptides have been identified that show great promise in AIS treatment, such as semax (heptapeptide Met-Glu-His-Phe-Pro-Gly-Pro), cortagen (tetrapeptide Ala-Glu-Asp-Pro), noopept (N-phenylacetyl-L-prolylglycine ethyl ester) and many others. And what is more the peptidergic mechanism of action is guessed for classical nootropic and neuroprotective agents such like piracetam [7]. Unfortunately, nearly all have failed to provide protection under the conditions of clinical use.

The literature data give evidence that some members of melanocortine peptides such as molecule of ACTH are capable to protect neuronal tissue from secondary damage such as inflammation and apoptosis and to recover injured by AIS brain tissue [8, 9, 10]. Particularly, the results showed that during AIS-associated inflammation, melanocortins such as homologous of ACTH sequences exert antipyretic effects by acting on melanocortine receptors (MCRs) located within the brain. [11]. It was shown that analogues of ACTH demonstrate neurotrophic properties and are capable of sustaining neurite outgrowth from cultured dorsal root ganglion and spinal cord cells in the absence of nerve growth factor [12].

Our attention was attracted by sequence 15-18 (Lys-Lys-Arg-Arg) of ACTH. The cleavage of proopiomelanocortine (POMK, peptide-precursor of ACTH) by prohormone converting enzyme and carboxypeptidases between two pairs of basic amino acids residues (-Lys-Lys-, -Arg-Lys-, -Arg-Arg-, -Lys-Arg-) leads to the generation of biologically active fragments [13]. Then it was found that for MCRs receptor binding and activation by ACTH, the amino acid residues 15-18 are the most important [14]. On their basis were synthetized 10 tetrapeptides, general formula acetyl-Lys-Lys-Arg-Arg-amide and were found their pronounced pharmacology properties.

Two peptides from these – KK-1 ((D-Lys)-Lys-Arg-Arg) and KK-5 ((D-Lys)-Lys-(D-Arg)-Arg) have shown promise exceeding of efficacy of known peptidergic drugs (semax and noopept) in our previous studies. It was shown that these peptides demonstrated antihypoxic properties under the conditions of normobaric hypoxic hypoxia with hypercapnia and asphyxia caused by clamping the trachea of rats [15, 16]. They reduced ND and cognitive deficit of rats with model of cerebral ischemia [17], improved indices of systemic and cerebral blood flow [18], decreased neurodestruction and neuroapoptosis in accordance to inhibiting of S-100 protein and neuron-specific enolase releasing into brain tissue [19]. Results of neuroprotective action of tetrapeptides KK-1 and KK-5 were verified histologically [20].

The research objective is to find out the novel neuroprotective oligopeptides KK-1 and KK-5 influence on the level of pro-inflammatory (TNF-α, IL-1β) and anti-inflammatory (IL-1β) cytokines, NGF level in the brain, as well as survival and ND severity at the model of AIS in rats.

2. Materials and Methods

2.1. Drugs and Chemicals

The objects of the research are the novel linear tetrapeptides, homologous aminoacids of a primary sequence of $ACTH_{15-18}$. Neuropeptides structure is (D-Lys)-Lys-Arg-Arg (D-Lys15-$ACTH_{15-18}$, KK-1) and (D-Lys)-Lys-(D-Arg)-Arg (D-Lys15, D-Arg17-$ACTH_{15-18}$, KK-5). Peptides were synthesized at State Research Institute of Highly Pure Biopreparations (Saint-Petersburg) by A. Kolobov, Doctor of Biology. The drug of comparison semax is the chemical and pharmacological analog of the investigated oligopeptides. It is the homologue of $ACTH_{4-7}$ stabilized by tripeptide Pro-Gly-Pro. The semax is used as intranasal droops for emergency in patients with AIS and as nootropic drug.

2.2. Ethical Guidelines

The experiment was carried out according to the requirements of European Communities Council Directive 2010/63/EU.

2.3. Animal Groups and Treatment

59 male rats, body mass equaled 180-220 grams were obtained from Central Research Laboratory of National University of Pharmacy vivarium (Kharkiv, Ukraine). Animals were kept according to the standard laboratory. Animals were divided randomly on such groups: 1 – sham-operated (SO, n=10), 2 – untreated group (UG, n=16), 3 – reference drug semax (n=11), 4 – KK-1 (n=12), 5 – KK-5 (n=10).

2.4. The Model of Ischemic Rats Injury and Biomaterial Preparation

The AIS model was caused by irreversible bilateral carotid occlusion under propofol anesthesia («diprivan», «Fresenius Kabi», Austria, 60 mg/kg intraperitoneal) [21]. Oligopeptides and semax were injected intranasally (i/n) as aqueous solutions at a dose of 0.02 mg/kg immediately after occlusion. In 24 hours rats survival were registered and ND was estimated according to McGraw Stroke Index scale [22].

The McGraw Stroke Index Scale is presented in table 1. The stroke index of each animal is the sum of points according to the neurological symptoms.

Table 1. *McGraw Stroke Index Scale.*

The neurologic symptom	Stroke index score
Paucity of movements	0.5
Tremor	1.0
Ptosis of one or both eyes	1.5
Weakness of limbs	1.5
Circling behavior	3.0
Paresis of 1-4 limbs	2.0-5.0
Paralysis of 1-4 limbs	3.0-6.0
Seizures	3.0
Extreme weakness (comatose)	7.0
Death	10,0

On 2nd day after neurological examination animals were anesthetized with chloroform, decapitated, the brain was immediately frozen in liquid nitrogen and stored at –70ºC until analysis.

2.5. Cytokines and NGF Determination

The level of cytokines and NGF was estimated by enzyme-linked immunosorbent assay (analyzer STAT FAX 303+, USA) using of beta-NGF ELISA-Kit sets (RayBiotech, Inc., USA), for the TNF-α and IL-4 cytokines – Cytokines (Russian Federation), for the IL-1β cytokine – Vector-Best (Russian Federation). Brain was homogenized into Potter-Elvehjem homogenizer at cooled 0.1 M tris-HCl buffer with 0.32 M of saccharose added (the ratio of 1:5, volume/volume). The homogenate was centrifuged during 15 minutes at 3000 rpm. The supernatant was used for analysis.

2.6. Statistical Analysis

All data with parametric distribution are presented as mean±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA). Fisher angular transformation of φ was used when the results were determined in alternative manner (presence or absence of characteristic). To determine the relationship between the individual parameters, the Spearman's correlation coefficient of ρ was used. The level of significance was defined as $p<0.05$.

3. Results

All sham-operated animals were alive without symptoms of ND (table 2) 24 hours after the carotid occlusion. In animals of the UG survival rate equaled 68.8% and in reference drug semax group it equaled 54.6% ($p>0.05$ compared with the SO group). Oligopeptides KK-1 and KK - 5 increased rats' survival after cerebral ischemia up to 75% and 80%, respectively ($p>0.05$ compared with the UG).

Table 2. *The survival rate and neurological deficit of rats after 24 hours of irreversible bilateral carotid occlusion.*

Indices	Group, drug, dose, administration route, n				
	SO, n=10	UG, n=16	Semax, 0.02 mg/kg i/n, n=11	KK-1, 0.02 mg/kg i/n, n=12	KK-5, 0.02 mg/kg i/n, n=10
Survival rate, %	100.0	68.8	54.6	75.0	80.0
Neurological deficit, point	0.15±0.08	2.14±0.43*	1,25±0.87*	1.67±0.79*	1,25±0.27*

Note. SO – the group of shame-operated animals, UG – the group of untreated animals, i/n – intranasal. *- Significant at $p<0.05$ compared with SO.

The survived UG animals were characterized by the expressive ND. Its symptoms were also manifested in the animals of other experimental groups. But severity of ND in these groups was significantly less compared with the index of UG. In particular, in the UG the following symptoms were registered: muscular weakness and lethargy, hyporeflexia, blepharoptosis, circling moves, paresis or paralysis of one or several extremities, coma. In animals that were treated with neuropeptides KK-1, KK-5 and drug of comparison semax the severity of ND decreased in 1.3, 1.7 and 1.7 times, respectively, compared with the analogous value of the UG ($p=0.62$). Hence the investigated peptides show the pronounced neuroprotective effect after single administration at the therapeutic regimen. This confirms the previously obtained results [15, 17].

The level of all cytokines and NGF was significantly increased in the brain of UG animals 24 hours after AIS (figure 1, 2, 3, 4). The level of NGF was increased by 5.6 times ($p=0.00013$), TNF-α – by 2.8 times ($p=0.00013$), IL-1β – by 2.3 times ($p=0.00013$), IL-4 – by 1.4 times ($p=0.00012$). Correlation analysis point to the lack of statically significant connection between the measured biochemical indices at the UG animals.

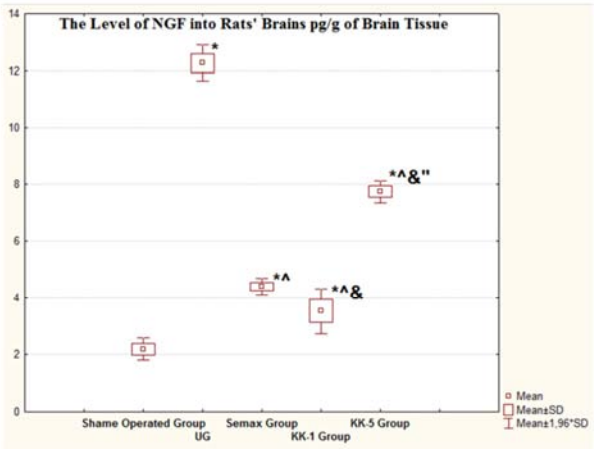

Figure 1. *The levels of NGF into rats' brains after 24 hours of irreversible bilateral carotid occlusion, pg/g of brain tissue. Note. Statistically significant at p<0.05: * – compared with SO, ^ - compared with UG, & – compared with semax group, " – compared with KK-1 group.*

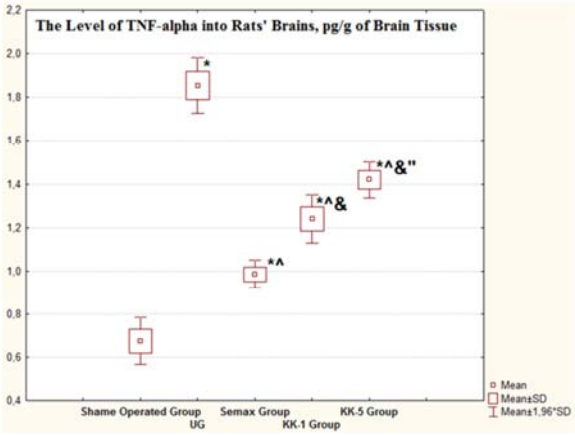

Figure 2. *The levels of TNF-alpha into rats' brains after 24 hours of irreversible bilateral carotid occlusion, pg/g of brain tissue.*

Note. Statistically significant at p<0.05: * – compared with SO, ^ - compared with UG, & – compared with semax group, " – compared with KK-1 group.

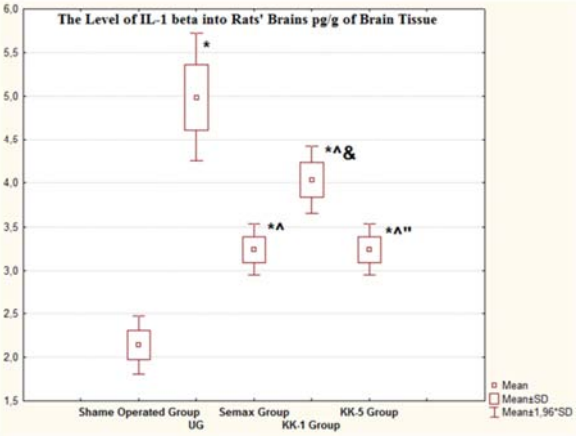

Figure 3. *The levels of IL-1 beta into rats' brains after 24 hours of irreversible bilateral carotid occlusion, pg/g of brain tissue.*

Note. Statistically significant at p<0.05: * – compared with SO, ^ - compared with UG, & – compared with semax group, " – compared with KK-1 group.

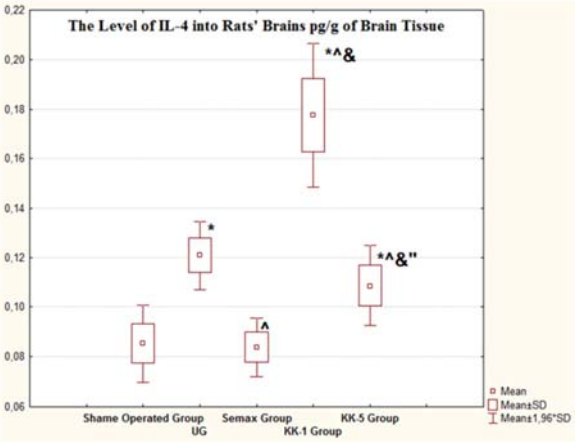

Figure 4. *The levels of IL-4 into rats' brains after 24 hours of irreversible bilateral carotid occlusion, pg/g of brain tissue. Note. Statistically significant at p<0.05: * – compared with SO, ^ - compared with UG, & – compared with semax group, " – compared with KK-1 group.*

Neuropeptides KK-1 and KK-5, as well as reference drug semax after single injection at therapeutic regimen at a dose of 0.02 mg/kg (i/n) normalized the level of all measured cytokines and NGF in rats' brains. But in these groups the cytokines and NGF level were still slightly elevated compared with the same indices of SO group animals.

The maximal decrease of NGF overexpression at the AIS model were observed after the neuropeptide KK-1 injection. The content of NGF in rats brain at the neuropeptide KK-1 injection is 3.5 times lower than the UG index (p=0.00012). After administration of the neuropeptide KK-5 and semax this index was statically significantly decreased by 1.6 and 2.8 times, respectively (p<0.001).

The level of TNF-α was maximally reduced under the influence of semax (by 1.9 times compared with the value of UG, p=0.00012) and IL-1β – under the action of semax and neuropeptide KK-5 (by 1.5 times for both medicines compared with the value of UG, p<0.001). Besides, correlation analysis reveals strong additional interconnection between decrease in NGF and decrease in TNF-α levels against a background of neuropeptide KK-5 (ρ=0.96, p<0.05, fig. 5).

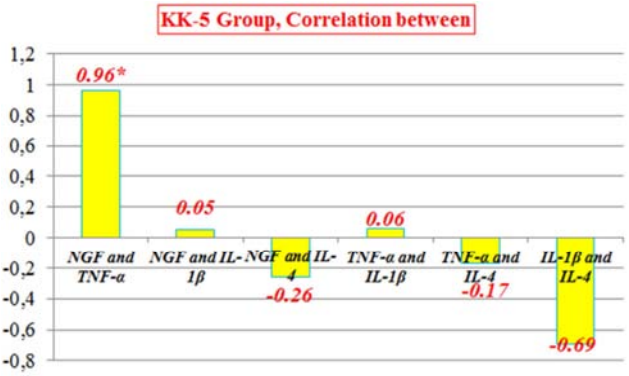

Figure 5. *The correlation between levels of NGF, TNF-α, IL-1β and IL-4 in animals of KK-5 group after 24 hours of irreversible bilateral carotid occlusion, * – statistically significant at p<0.05.*

Intranasal administration of neuropeptide KK-1 also caused a statistically significant decrease in the level of pro-inflammatory cytokines (TNF-α and IL-1β). It reached 66.7% and 81% of the UG animals value respectively (p<0.002). The tendency to the appearance of a moderate positive correlation (ρ=0.54, p>0.05) between decrease in NGF and decrease in TNF-α levels affected by using of the neuropeptide KK-1.

The investigated neuropeptides exerted a different influence on the content of the anti-inflammatory cytokine IL-4 into rats' brains at the model of AIS. So, semax and KK-5 decreased its level and neuropeptide KK-1 increased it. Under the influence of semax the level of IL-4 was normalized reaching the SO group value. Its ability to reduction demonstrate tendency to correlation with decrease in NGF level (ρ=0.66, p>0.05). The neuropeptide KK-5 reduced the content of IL-4 into rats' brains, on 9%, that was statistically significant compared with the UG value (p=0.0102). The treatment of rats with AIS model by

neuropeptide KK-1 led to the increase in IL-4 level into brain by 1.5 times compared with the UG value ($p<0.00012$). Besides, was shown tendency to negative correlation relationship NGF and IL-4 levels decrease after injection of KK-1 ($\rho = -0.59$, $p>0.05$).

4. Discussion

Hence, the obtained results prove the neuroprotective effect of the tetrapeptides KK-1 and KK-5 at the model of AIS in rats. It is evidenced by the increase in survival rate and reduction of ND severity. The influence of the peptides on the expression of pro-inflammatory and anti-inflammatory cytokines and NGF can be considered as an important link in the mechanisms of their neuroprotective activity.

The data in literature evidence that the main producers of NGF (hippocampus and some other anatomical formations) and its consumer-cells in brain form the dynamic structure that operates according to the functional needs of the central and peripheral nervous system morphogenesis [23]. This system is the first react to the surrounding stimuli, especially to the injury. The reaction of the increased NGF synthesis of the in response to neuronal ischemia is present as both reactive and reparative phase of the endogenous neuroprotective process [24]. It has been established that the level of NGF in the core ischemic zone and penumbra zone during one week of the ischemia period at the AIS is decreased dramatically [25]. But cerebral tissues that avoid ischemia react on the damaging factor by the NGF overexpression (reactive increasing of synthesis). The role of this factor is intensification of the functional activity of neurons that have survived (preparation to the neuroplastic transformations) [26]. Thus release of NGF after AIS is a process intended for providing of brain homeostasis overpronounced in comparison with normal conditions [27]. Decrease of this neurotrophic factor level observed after the treatment of rats with AIS model by neuropeptides KK-1 and KK-5 indicate the reduction in the destructive processes which are the triggers of synthesis and secretion of the NGF by neurons. The results of our research (modulation of NGF expression at the AIS by neuropeptides, homologouses of sequence of $ACTH_{15-18}$) are confirmed by previously obtained data results about the important role of the ACTH and its fragments in regulation of the NGF trophic function and other neurotrophic factors obtained previously [28, 29].

The obtained results indicate the supplementary mechanism of tetrapeptides KK-1 and KK-5 neuroprotective activity that includes reduction of TNF-α level in brain after AIS. Strong interconnection between decrease in levels of NGF and TNF-α after administration of the neuropeptide KK-5 has been found. It should be noted that both mentioned neuronal agents may lead to the neurons death trough apoptosis with the mediation of the similar signal channel. The latter is connected with homodimer of the low-affinity p75 neurotrophin receptor (p75NTR), the ligands of which are both NGF and TNF-α. Stimulation of homodimer p75NTR induces phosphorylation of pro-apoptic early response proteins c-fos and c-jun leading to programmed cell death [30, 21]. But there is an alternative signal stage for the NGF. It is realized when NGF binds to the heterodimeric form of p75NTR or to another class of receptors such as tropomyosin receptor kinase A (TrkA) [32]. It leads to the release of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and further proliferation of the neuronal axons. Intracellular adaptation of this process is provided by the member of TNF family – TRAF6 [33]. Direction of the endogenous neuroprotective process by alternate way (way of survival and further proliferation of neurons) can be explained by the decreased concentration of the ligand (NGF) that leads to its interaction mainly with high-affinity receptor of the neurotrophic TrkA and heterodimeric form of p75NTR. Low concentration of TNF-α in turn suppresses the process of the penumbra neurons programmed cell death due to expression of c-fos and c-jun [34]. This assumption can explain the role of mutual reduction of NGF and TNF-α levels in survived animals and ND reduction after administration of neuropeptides KK-1 and KK-5.

Reducing of TNF-α and IL-1β level and increasing of IL-4 expression characterize anti-inflammatory activity of the investigated neuropeptides. The result of the decreased IL-1β synthesis in the injured part of the brain after AIS is the reduction of the reactive NO-synthase and cyclooxygenase level, the decrease in the circulating effector cells adhesion, reduction of excitotoxic damage of the injured brain tissue and, finally, – reduction of the core ischemic zone [35, 36, 37]. Reduction of TNF-α and IL-1β expression is the positive predictive index of the AIS course in clinic [38].

Anti-inflammatory activity of neuropeptide KK-1 is confirmed by statically significant increase in IL-4 level compared with the UG and accompanied with the tendency to negative correlation with decrease of this value in NGF level ($\rho = -0.59$, $p>0.05$). The IL-4 is one of the main anti-inflammatory cytokines in brain at cerebral ischemia and other injuries of the CNS [13]. It is generated by CD4+ T-lymphocytes (Th2), eosinophils and mast cells. Its anti-inflammatory activity is directed to the suppression of differentiation of CD4+ T- lymphocytes population of Th1, suppression of the macrophages ability to generate IL-1, NGF and interleukine-6, increase in peroxisome proliferator-activated receptor gamma (PPARγ) expression etc. [39, 40].

According to the all mentioned data, the effect of the investigated neuropeptides on cytokine system of rats brain at the model of AIS should be underlined. The established neurotrophic and anti-inflammatory action of neuropeptides mediates their neuroprotective effect and is revealed by increase in survival rate and ND decrease in rats with AIS.

As it follows from the results, the investigated peptides demonstrate some benefit comparing with traditional treatment of AIS using semax. It's related to higher survival rate of rats provided by peptides KK-1 and KK-5 and different profile of influence on the level of cytokines under the conditions of model AIS. Particularly, as it shown in the fig. 1, peptide KK-1 reduced overexpression of NGF more

pronounced than reference drug semax (p=0.00012). Furthermore, the level of the basic anti-inflammatory cytokine IL-4 was increased by KK-1 maximally (fig. 4), compared with semax and KK-5 groups. From the point of view of authors, these subtle differences of mechanisms of neuroprotective activity of peptides from traditionally used $ACTH_{4-7}$ analogue semax can provide their higher efficacy established in our previous studies.

In our opinion it is necessary to continue the research of safety and effectiveness of the neuropeptides KK-1 and KK-5 for the purpose of new neuroprotective drugs development.

5. Conclusions

Tetrapeptides KK-1 and KK-5 that are homologous of $ACTH_{15-18}$ show neuroprotective activity at the model of cerebral ischemia in rats. It is confirmed by the increase in survival rate up to 75% and 80%, respectively and also reduction of the neurological deficit by 1.5 times.

It has been established that the mechanism of the tetrapeptides KK-1 and KK-5 neuroprotective activity is connected with their neurotrophic and anti-inflammatory action. Intranasal administration of the neuropeptides to rats with cerebral ischemia leads to the decrease in overexpression of the nerve growth factor (NGF), tumor necrosis factor α (TNF-α), interleukine-1 beta (IL-1β) and interleukine-4 (IL-4).

Positive correlation between reduction of overexpression of the NGF and decrease in TNF-α after administration of the neuropeptides KK-1 and KK-5, statistically significant and in the form of tendency, respectively.

The peptide KK-1 increases expression of IL-4 in rats with cerebral ischemia in contrast to the reference drug semax, as well as neuropeptide KK-5. The tendency to negative correlation between decrease in level of NGF and reduction in IL-4 content after usage of this neuropeptide has been shown.

Further research of safety and effectiveness of neuropeptide KK-1 and KK-5 for purpose of the new neuroprotective medical product development is expedient.

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