

# The effect of intranasal administration of an IL-1b antagonist (RAIL) on the state of the nitroxydergic system of the brain during modeling of acute cerebrovascular accident

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## Abstract

**Introduction:** This study was designed to evaluate the effect activity of RAIL-gel in comparison with Citicoline on nitroxydergic system during acute cerebrovascular accident.

**Methods:** In this study, 80 white nonlinear rats were randomly assigned to 4 groups (20 rats in each): 1) intact; 2) control - untreated with acute cerebrovascular accident (ACVA), examined on the 4<sup>th</sup> day; 3) animals with ACVA, receiving RAIL, examined on the 18<sup>th</sup> day; 4) animals with ACVA, treated with Citicoline, examined on the 4<sup>th</sup> day. The expression of inducible NOS was determined by Western blotting. The nitrosative stress marker, nitrotyrosine, was determined using the ELISE kit NITROTYROSINE (kit no. HK501-02, series 12825k1212-k). To assess the state of iNOS mRNA expression, we used the method of polymerase chain reaction with reverse transcription in real time (RT-PCR).

**Results:** Our research demonstrated that course administration of the RAIL and Citicoline to animals with ACVA for 4 days leads to the stabilization of the parameters of the brain nitroxydergic system. However, Citicoline does not provide a full effect on the shifts of the NO system in the brain. It does not have the proper effect on such an important link in the pathogenesis of ischemic brain damage as nitrosative stress. RAIL leads to a significant decrease in NOS activity due to its inducible form (decrease in the expression of iNOS and iNOS mRNA) and a decrease in NO metabolites, and inhibition of nitrosative stress (decrease in nitrotyrosine).

**Conclusion:** IL-1b antagonist RAIL (Intranasal Gel) significantly exceeds Citicoline in terms of the severity of the effect on the nitroxydergic system indicators.

## Keywords

Citicoline, nitrosative stress, iNOS, NOS, nitrotyrosine, nitrites

## Introduction

Improving aid measures, incl. medication, in acute disorders of cerebral circulation is an important task of health care. The effectiveness of neuroprotective therapy completely depends on the correct target link for ischemic neurodestruction. ACVA launches a complex staged cascade of molecular biochemical reactions of neuronal damage, including energy deficit, lactate acidosis, transmitter autotoxicity, oxidative stress and neuroinflammation. In this case, a closed “vicious” circle is formed- inflammation leads to the activation of sensitive to redox potential signal transduction pathways (MAPK, NFkB), which increases oxidative stress. Significant role in mechanisms of neuronal death during the development of calcium glutamate cascade belongs to NO-mediated mechanisms, which are realized due to activation of iNOS. Initially, during calcium glutamate cascade an activation of nNOS and overproduction of NO, which is involved in damage to neurons is observed. Its toxic effect is associated with a violation of mitochondrial oxidative phosphorylation and metabolism of ribonucleotide reductase, and formation of highly toxic ONOO<sup>-</sup>, which blocks a number of neuronal receptors, inactivates SOD and causes aggravation of ROS-dependent damage to nerve tissue. During late stages (after 24 hours after modeling stroke in rats (Chekman et al. 2016; Belenichev et al. 2020b) there is an increase in iNOS expression, which enhances NO-dependent mechanisms of neurodestruction. An important role in the regulation of iNOS expression and activation of nitrosative stress in cerebral ischemia belongs to proinflammatory cytokines, especially IL-1b. Thus, IL-1b activates AP-1 and NF-kB, which, under conditions of ischemia, change the cellular signaling and enhance the expression of other pro-inflammatory factors, stimulate the expression of iNOS by astrocytes (Belenichev et al. 2008, 2015). An excess of IL-1b can negatively affect the transport of reduced glutathione and reduce its synthesis. Deficiency of intracellular glutathione, which is involved in the mechanisms of NO transport and its bioavailability, enhances the formation of ONOO<sup>-</sup> (Belenichev et al. 2008). The well-known role of IL-1b in the modulation of HSP70 expression is from activation to inhibition, depending on the concentration (Suprun 2011; Chekman et al. 2016). One of the interesting and developing research areas of rational neuroprotection is the interruption of IL-1b - mechanisms of neurodestruction by specific antagonists of IL-1b (Lundblad and Macdonald 2010). At present, convincing data have been obtained on the neuroprotective effect of the IL-1b antagonist, RAIL (Belenichev et al. 2015). However, the mechanism of this action is not fully understood. Its effect on disorders of the nitroxydergic system of the brain during ACVA was not revealed. Pharmacologists and clinicians are also interested in a new dosage form of RAIL - a gel for intranasal use. All of the above determines the relevance of this study.

**The purpose** of this work is to study the effect of RAIL gel at intranasal use at nitroxydergic system in the brain of rats in acute cerebrovascular accident

## Materials and research methods

The experiments were carried out on 80 white outbred rats weighing 170–180 g, of both sexes, obtained from the nursery of the GA “Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine”. The duration of quarantine (acclimatization period) for all animals was 14 days. During the quarantine, each animal was examined daily (behavior and general condition), also animals were observed in cages twice a day (morbidity and mortality). Before the start of the study, animals meeting the criteria for inclusion in the experiment were assigned to groups using a randomization method. Animals that did not meet the criteria were excluded from the study during quarantine. The animal cages were placed in separate rooms. Light mode: 12 hours - light, 12 hours - dark. The air temperature was maintained within 19–25 °C, relative humidity - 50–70%. Air temperature and humidity were recorded daily. The ventilation mode was set, providing about 15 room volumes per hour. Experimental animals were kept on the same rations, under normal vivarium conditions (Mironov and Bunatyan 2012). The animals were housed in standard cages: rats - 5 animals per cage, rabbits - 1 animal per cage. Diet - feed grains, fish, cottage cheese, bread, root crops (beets, carrots). All manipulations were carried out in accordance with the regulation on the use of animals in biomedical experiments (Strasbourg 1986, as amended in 1998). Experimental research protocols and their results were approved by the decision of the ZSMU Commission on Bioethics (Protocol No. 32 dated October 26, 2018). A model of acute cerebrovascular accident (ACVA) by the type of ischemic stroke was used (Chekman et al. 2016). This model was reproduced by bilateral occlusion of the common carotid arteries in Wistar rats. Considering the high mortality rate for this experimental model, such a number of animals were operated on so that by the 4<sup>th</sup> day there were 20 animals in each group. The operation was performed under thiopental anesthesia (40 mg / kg). Animals of different groups were taken out of the experiment on the 4<sup>th</sup> day of observation under thiopental anesthesia (40 mg / kg). The studied drugs were administered according to the scheme: RAIL - intranasally in the form of a gel (1 mg / kg) and the reference drug Citicoline - intraperitoneally (500 mg / kg) (Belenichev et al. 2008, 2015). Citicoline has been approved and widely used for over 30 years in Western Europe and the United States for the treatment of cerebral stroke (ischemic and hemorrhagic) and TBI. Citicoline produced by Ferrer International S.A., Spain and represented by Nycomed Austria GmbH, Austria was used in this research.

In this series of experiments there were four groups of animals:

- 1) intact - (20 rats);
- 2) control - untreated with acute cerebrovascular accident (ACVA), examined on the 4<sup>th</sup> day (20 rats);
- 3) animals with ACVA, receiving RAIL, examined on the 18<sup>th</sup> day (20 rats);
- 4) animals with ACVA, treated with Citicoline, examined on the 4<sup>th</sup> day (20 rats).

We used the RAIL substance obtained from the Federal State Unitary Enterprise “State Research Institute of Highly Pure Biological Preparations” (Russia, S-Pererburg, LSR-007452 / 1-0300710). The substance was obtained biotechnologically from *E. coli* TG1 (pTAC- hIL-1ra), consists of 153 amino acids. M.m. 17, 906 kDa. RAIL-gel (5 mg / 1 ml) was developed at the Department of Medicines Technology, ZSMU. At the end of the experiment, the animals were withdrawn from the experiment after 2–4 minutes. after injection of sodium thiopental (40 mg / kg) (before the loss of the straightening reflex) in order to minimize neurometabolic shifts. Blood was quickly removed from the brain, brain was separated from the meninges, and the test pieces were ground in liquid nitrogen to a powdery state and homogenized in a 10-fold volume of medium at (2 °C) containing (in mmol): sucrose - 250, Tris-HCl- buffer - 20, EDTA -1 (pH 7.4). Mitochondrial and cytosolic fractions were isolated at a temperature (+ 4 °C) by differential centrifugation in a Sigma 3–30k refrigerated centrifuge (Germany). (20 minutes at 17000 g). To assess the state of the nitroxydergic system, the NOS activity and the level of nitrites were determined biochemically. The expression of inducible NOS was determined by Western blotting. Proteins were separated on a 10% polyacrylamide gel by electrophoresis. Used primary antibodies against iNOS (Santa Cruz Biotechnology) and secondary (biotinylated anti-mouse IgG, SIGMA, USA, cat. # 051M4885). The nitrosative stress marker, nitrotyrosine, was determined using the ELISE kit NITROTYROSINE (kit no. HK501-02, series 12825k1212-k). To assess the state of iNOS mRNA expression, we used the method of polymerase chain reaction with reverse transcription in real time (RT-PCR). To prepare biomaterial samples, pieces of the brain were placed in a Bouin fixative for a day and, after standard histological dehydration, the tissue was embedded in paraffin. On a rotary microtome, 5-micron thick sections of the CA-1 zone of the hippocampus were cut. After dewaxing and centrifugation, the precipitate was dried in air to remove residual ethanol. Isolation of total RNA from rat tissue was performed using the Trizol RNA Prep 100 kit (IZOGEN, Russia). For reverse transcription (synthesis to DNA), a Reagent Kit for Reverse Transcription (OT-1) (SINTOL, Moscow) was used. To determine the expression level of the genes under study, a CFX96 Real-Time PCR Detection Systems amplifier (Bio-Rad Laboratories, Inc., USA) and a set of reagents for RT-PCR in the presence of SYBR Green R-402 (Syntol, Russia). Specific primer pairs (5'-3') for the analysis of the studied and reference genes were selected using the PrimerBlast software ([www.ncbi.nlm.nih.gov/tools/primer-blast](http://www.ncbi.nlm.nih.gov/tools/primer-blast)) and manufactured by ThermoScientific, USA. The registration of the fluorescence intensity took place automatically at the end of the elongation stage of each cycle through the SybrGreen channel automatically. The actin, beta (Actb) gene was used as a reference gene to determine the relative value of changes in the expression level of the studied genes. Protein concentration was estimated by the Bradford method.

The research results were processed using the statistical package of the licensed program “STATISTICA for Windows 6.1” (StatSoft Inc., No. AXX R712D833214SAN5), as well as “SPSS 16.0”, “Microsoft Excel 2003”. The significance of differences between the experimental groups was carried out according to the Whitney-Mann test.

## Results and discussion

As a result of the conducted studies, it was found that modeling of ACVA leads on the 4<sup>th</sup> day of the experiment to disruption of the nitroxydergic system and initiation of nitrosative stress in the brain of experimental animals, as evidenced by a significant increase in the cytosolic and mitochondrial fractions of the brain homogenate of animals in the NOS activity by 439.5% and 189.2%, nitrites by 157.7% and 96.1%, as well as nitrotyrosine by 162% and 66.1%, respectively (Tables 1 and 2).

**Table 1.** Indicators of the nitroxydergic system and nitrosative stress in the cytosolic fraction of the brain homogenate of rats with ACVA on the 4<sup>th</sup> day of the experiment.

Group of animals	NOS nmol / mg protein / min	Nitrites µM / g / protein	Nitrotyrosine nmol / mg / protein	iNOS, c.u. / g protein
Sham-operated	2.43 ± 0.11	4.12 ± 0.35	18.4 ± 1.11	0.12 ± 0.01
animals with ACVA (control)	13.11 ± 1.10 <sup>+</sup>	10.62 ± 0.91 <sup>+</sup>	48.2 ± 2.87 <sup>+</sup>	0.44 ± 0.05 <sup>+</sup>
animals with ACVA + Citicoline	9.42 ± 0.62 <sup>++</sup>	8.97 ± 0.34 <sup>+</sup>	34.1 ± 2.21 <sup>++</sup>	0.45 ± 0.04 <sup>+</sup>
animals with ACVA + RAIL	4.75 ± 0.23 <sup>*+1</sup>	5.71 ± 0.41 <sup>*1</sup>	23.2 ± 1.43 <sup>*+1</sup>	0.23 ± 0.01 <sup>*1</sup>

Note: \* - p ≤ 0.05 in relation to the control group;

+ - p ≤ 0.05 in relation to the group of sham-operated animals;

<sup>1</sup>- p ≤ 0.05 in relation to the Citicoline group.

**Table 2.** Indicators of the nitroxydergic system and nitrosative stress in the mitochondrial fraction of the brain homogenate of rats with ACVA on the 4<sup>th</sup> day of the experiment.

Group of animals	NOS nmol / mg protein / min	Nitrites µM / g / protein	Nitrotyrosine nmol / mg / protein	iNOS, c.u. / g protein
Sham-operated	1.11 ± 0.03	1.81 ± 0.08	5.6 ± 0.44	0.077 ± 0.005
animals with ACVA (control)	3.21 ± 0.21 <sup>+</sup>	3.55 ± 0.20 <sup>+</sup>	9.3 ± 0.32 <sup>+</sup>	0.17 ± 0.001 <sup>+</sup>
animals with ACVA + Citicoline	2.11 ± 0.11 <sup>++</sup>	3.11 ± 0.32 <sup>+</sup>	7.5 ± 0.42 <sup>++</sup>	0.12 ± 0.001 <sup>++</sup>
animals with ACVA + RAIL	1.53 ± 0.08 <sup>*</sup>	2.32 ± 0.17 <sup>**</sup>	6.1 ± 0.32 <sup>++</sup>	0.08 ± 0.002 <sup>*+1</sup>

Note: \* - p ≤ 0.05 in relation to the control group;

+ - p ≤ 0.05 in relation to the group of sham-operated animals;

p ≤ 0.05 in relation to the Citicoline group.

A significant increase in the concentration of iNOS in the cytosol by 267% and by 120.7% in the mitochondria of the brain of animals with ACVA was also found. Analyzing the data presented in the table characterizing the expression of iNOS mRNA in the CA1-zone of the hippocampus of the brain of rats with ACVA on the 4<sup>th</sup> day of

**Table 3.** Expression indices of iNOS mRNA in the CA1-zone of the hippocampus with ACVA on the 4<sup>th</sup> day of the experiment.

Group of animals	Expression of iNOS mRNA, c.u.	
	relatively sham-operated group	Relatively control
Sham-operated animals with ACVA (control)	1.00 ± 0.011	-
animals with ACVA + Citicoline	7.77 ± 0.011 <sup>+</sup>	0.82 ± 0.001 <sup>*</sup>
animals with ACVA + RAIL	2.19 ± 0.009 <sup>+1</sup>	0.057 ± 0.0008 <sup>*1</sup>

Note: \* -  $p \leq 0.05$  in relation to the control group;

<sup>+</sup> -  $p \leq 0.05$  in relation to the group of sham-operated animals;

<sup>1</sup> -  $p \leq 0.05$  in relation to the Citicoline group.

the experiment, the following was found. The expression of pNOS mRNA and, especially, iNOS mRNA in the treated groups was higher than the values of sham-operated animals but lower than the control values (Table 3). It can be concluded that a significant increase in the expression of iNOS mRNA is observed on the 4<sup>th</sup> day after ACVA. The data obtained are in line with the modern concept of neurodegeneration during cerebral ischemia, with which our previous studies are consistent, which demonstrate a significant increase in NO production due to an increase in the activity and expression of nNOS (12–24 hours after occlusion of the carotid arteries) and iNOS (starting from 1 day after occlusion of the carotid arteries) (Mongin et al. 2012). It is well-known that nitrosative stress leads to overproduction of cytotoxic derivatives of NO - nitrosonium ion, peroxy nitrite, which attack protein molecules, forming 6-nitrotryptophan, 3-nitrotyrosine, 3-chloro tyrosine, 2-oxohistidine, as well as various carbonyl derivatives (Kolesnik et al. 2013). N-, S-nitrosation of protein fragments of neuronal membranes impairs the sensitivity and specificity of receptors, generation, formation and transmission of nerve impulses, disrupts synaptic transmission (Bardag-Gorce et al. 2010; Ruland 2011; Zhang et al. 2011; Aquilano et al. 2014; Chekman et al. 2016). In addition, cytotoxic derivatives of NO disrupt the permeability of the mitochondrial pore and are involved in the formation of mitochondrial dysfunction (Zhou and Iadecola 2007; Belenichev et al. 2015, 2020b; Chekman et al. 2016).

The course administration of Citicoline intraperitoneally for 4 days at a dose of 500 mg / kg to animals with ACVA led to a significant decrease NOS activity in the cytosol and mitochondria of the brain by 28.1% and 34.2%, nitrotyrosine by 29.2% and 19.3%, respectively (Tables 1 and 2). Citicoline led to a significant decrease in the expression of iNOS in the mitochondria of the brain by 29.4%. The introduction of Citicoline also led to a significant decrease in the expression of iNOS mRNA by 18% in the CA1 zone of the hippocampus relatively data of the group. In this case, the expression of iNOS mRNA in the CA1 zone of the hippocampus of rats with ACVA treated with Citicoline was 7.7 times higher than in the sham-operated group. (Table 3). It should be noted that by the severity of the effect on such indicators as NOS activity, the concentration of nitrites and nitrotyrosine in the cytosolic fraction of the brain, as well as on the concentration of iNOS in the mitochon-

dria of experimental animals' brain homogenate, it is significantly inferior to RAIL. Citicoline is also significantly inferior to RAIL in its influence on the expression of iNOS mRNA in the CA1 hippocampus. The Citicoline mechanism of action is well studied (Zhou and Iadecola 2007; Belenichev et al. 2008, 2015). The decrease in iNOS expression under the action of Citicoline can be explained not only by its ability to reduce the formation of ROS by the energetic reactions of mitochondria involved in its expression (Zhao et al. 2007; Boche et al. 2013; He et al. 2017; Tschoe et al. 2020). We, in other studies, found that Citicoline exhibits a mitoprotective effect, preserves the integrity of the inner mitochondrial membrane, affecting the level of cardiolipin. Citicoline is able to increase the fund of intramitochondrial glutathione and, due to this glutathione, possibly interrupt the IL-1b-dependent mechanisms of expression of this iNOS (Zhou and Iadecola 2007). We also found that Citicoline inhibits the reactions of initiation of neuroapoptosis (He et al. 2017). However, analyzing the experimental data on the effect of Citicoline on the parameters of the nitroxydergic system of the rats' brain with ACVA, it can be concluded that the above mechanisms of its action do not provide a full effect on the shifts of the NO system in the brain. Thus, Citicoline, as a widely used neuroprotector, does not have an adequate effect on such an important link in the pathogenesis of ischemic brain damage as nitrosative stress, which requires additional pharmacological action on this target link to increase the effectiveness of complex therapy of cerebral strokes (Zhao et al. 2007; Shichita et al. 2012; Boche et al. 2013; Yuan et al. 2016; Tschoe et al. 2020). The course administration of RAIL gel intranasally for 4 days at a dose of 1 mg / kg to animals with ACVA led to a significant decrease in the activity of NOS by 63.7% and 52.3%, nitrites by 46.2% and 34.4%, nitrotyrosine by 51.8% and 34.4%, respectively (Tables 1 and 2) in the cytosol and mitochondria of the brain. RAIL resulted in a decrease in the expression of iNOS in the cytosol and mitochondria of the brain by 47.7% and 52.9%, respectively. The introduction of RAIL also led to a decrease in the expression of iNOS mRNA in the CA1 zone of the hippocampus by 94.3% compared to the control group. At the same time, the values of iNOS mRNA in the CA1 zone of the hippocampus in this group were 119% higher than in the group of sham-operated animals (Table 3). Intranasal use of RAIL contributes to a higher neuroavailability compared with intramuscular and intraperitoneal administration ([Suprun 2011) and provides a more pronounced pharmacological effect. The revealed effect of RAIL demonstrates its pronounced effect on the main indicators of disorders of the brain nitroxydergic system and the formation of nitrosative stress in cerebral ischemia. Such an effect can be provided, first of all, by the blockade of IL-1b (Belenichev et al. 2014a, b; Belenichev and Gorbacheva 2015), interruption of IL-1b - dependent pathway of iNOS expression and the cascade of nitrosative stress reactions triggered by this enzyme (Nefedov et al. 2016). The revealed facts of suppression of iNOS and iNOS mRNA expression in the ischemic brain by IL-1b antagonists put forward neuroinflammation, cytokines and IL-1b

as one of the leading causes (after excitotoxicity, mitochondrial dysfunction (Belenichev et al. 2017)) of NO overproduction, nitrosative stress and realization of neuronal death delayed mechanisms after stroke. In previous studies, we established the effect of RAIL on the expression of the heat shock protein 70 kDa in the sensorimotor zone neurons of the rat brain after carotid arteries occlusion on the 4<sup>th</sup> day (Suprun 2011; Chekman et al. 2016; Belenichev et al. 2018). It is known that HSP70 can lead to both an increase in IL-1 $\beta$  to the level required for participation in cyto- and neuroprotection, as well as suppress the expression of IL-1 $\beta$  (Belenichev et al. 2008, 2015, 2020a). HSP70 can prevent the production of inflammatory cytokines by interfering with NF- $\kappa$ B-dependent transcription (Ruland 2011). Therefore, we hypothesize the possibility of RAIL influencing through an increase in the HSP70 concentration (Vidale et al. 2017) on the activation of redox-sensitive transcription factors AP-1, NF- $\kappa$ B, NF-1 b and then on the expression of iNOS.

## Conclusions

1. Experimental modeling of ACVA leads to the initiation of nitrosative stress in the brain of experimental animals on the 4<sup>th</sup> day, as evidenced by an increase in NOS activity due to its inducible form (increased

expression of iNOS and iNOS mRNA), the content of stable NO metabolites and nitrotyrosine.

2. Course introduction for 4 days to animals with ACVA Citicoline (500 mg / kg) leads to a significant change in some parameters of the nitroxidergic system of the brain. However, Citicoline does not provide a full effect on the shifts of the NO system in the brain. It does not have the proper effect on such an important link in the pathogenesis of ischemic brain damage as nitrosative stress.
3. Course administration of the IL-1b receptor antagonist - RAIL intranasally to animals with ACVA for 4 days leads to the normalization of the parameters of the nitroxidergic system of the brain in varying degrees of severity of nitroxidergic system stabilization; a significant decrease in NOS activity due to its inducible form (decrease in the expression of iNOS and iNOS mRNA) and a decrease in NO metabolites, and inhibition of nitrosative stress (decrease in nitrotyrosine).
4. In terms of the severity of the effect on the nitroxidergic system indicators, RAIL significantly exceeds Citicoline.
5. The results obtained experimentally substantiate the prospects for further studies of the neuroprotective effect of the IL-1b receptor antagonist - RAIL in the form of the intranasal gel.

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